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Examination of performance of glassy carbon paste electrode modified with gold nanoparticle and xanthine oxidase for xanthine and hypoxanthine detection

Meliha Çubukçu^a, Suna Timur^b, Ülkü Anik^{a,*}

^a Mugla University, Faculty of Arts and Science, Chemistry Department, 48000 Kötekli, Mugla, Türkiye

^b Ege University, Faculty of Science, Biochemistry Department, 35100 Bornova, Izmir, Türkiye

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Abstract

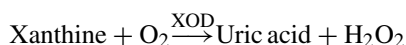
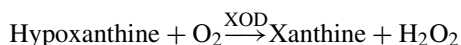
A composite electrode was prepared by modifying glassy carbon microparticles with gold nanoparticles (Au-nps) and xanthine oxidase enzyme (XOD) for xanthine (X) and hypoxanthine (Hx) detection. After the optimization of the system for X, the biosensor was characterized for X and Hx. A linearity was obtained in the concentration range between 5.00×10^{-7} and 1.00×10^{-5} M for X with equation of $y = 0.24x + 0.712$ and 5.00×10^{-6} to 1.50×10^{-4} M for Hx, with equation of $y = 0.014x + 0.575$, respectively. Obtained results were compared to X and/or Hx biosensors including/not including Au-np in the structure. The developed system was also applied for detection of Hx in canned tuna fish sample and very promising results were obtained.

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1. Introduction

The development of a sensor for xanthine (X) and hypoxanthine (Hx) is of medical and biological importance [1,2]. On the other hand, the levels of these compounds are generally used in the food industry as an index for evaluating meat or fish freshness [3]. Methods like chromatography and spectrophotometry were utilized for the detection of X and Hx [4,5]. However, since these methods are costly and laborious, alternative systems like amperometric biosensors have been developed for this particular application. In these systems, the level of X or Hx is monitored electrochemically by following H_2O_2 and/or uric acid oxidation or O_2 consumption produced by the XOD-catalyzed reaction depending on the charge of applied potential [6].



Recent developments about attractive properties of nanoparticles increase interest about their electrochemical behavior and applicability of these materials in biosensing areas [7]. Gold nanoparticle (Au-np) is a kind of nanomaterial where it can help proteins to retain their biological activity upon adsorption [8,9]. Its large surface area and good electronic properties [10] and its utilization for the study of direct electron transfer of redox proteins [10–12] increase their usage in biosensor construction [12–14].

Pingarron and co-workers [6] have used Au-nps for various kinds of sensors and biosensors. They prepared glucose oxidase (GO_x) biosensors by immobilizing GO_x onto different tailored Au-nps modified electrode surfaces [15]. In another work they obtained colloidal gold–carbon paste electrodes (CPEs) by using CPEs modified with cysteamine SAMs [16]. Recently, a xanthine oxidase (XOD) biosensor, based on a carbon paste electrode (CPE) modified with electrodeposited Au-nps, for the amperometric determination of hypoxanthine (Hx) is reported by the same group [6].

Another novel material that was used for X and Hx biosensor construction is glassy carbon microparticles. Combination

* Corresponding author. Tel.: +90 252 2111503.

E-mail address: ulkuanik@yahoo.com (Ü. Anik).

of this material with mineral oil results with the formation of glassy carbon paste electrode (GCPE). It has been demonstrated that this electrode has better electrochemical reactivity towards the oxidation of hydrogen peroxide compared to conventional CPE [17]. In our previous work, GCPE was modified with xanthine oxidase enzyme (XOD) to obtain X and Hx biosensor and very promising results with low detection limit were obtained [18].

In the present work, for the first time GCPE was modified with Au nanoparticles and XOD for obtaining proper composite structure that can be used as transducer for X and Hx biosensor. After the optimization of the system with X, the biosensor was applied for Hx detection in canned tuna fish. The performance of the biosensor was also compared with other X and Hx biosensors.

2. Experimental

2.1. Apparatus

Chronoamperometric experiments were carried out with the AUTOLAB PGSTAT 12 electrochemical measurement system from ECO CHEMIE Instruments B.V. (The Netherlands) driven by GPES software. The experiments were conducted in a voltammetric cell (Metrohm), at room temperature (25 °C), using a three-electrode configuration. A platinum electrode was served as an auxiliary electrode and an Ag/AgCl as a reference electrode (Metrohm). Electrodes were inserted into the cell through its Teflon cover.

2.2. Reagents and materials

Xanthine oxidase from buttermilk (XO, 0.06 units/mg solid) was obtained from Sigma. Glassy carbon spherical powder (20–50 µM) was purchased from Alfa Aesar, while mineral oil was obtained from Aldrich and gold colloid from Sigma (0.75 A₅₂₀ units/mL; 10 nm ~0.001% as HAuCl₄). Phosphate buffer (50.0 mM, pH 7.5) was served as supporting electrolyte. One millimolar Hx and X solutions were prepared freshly by dissolving the appropriate amount of Hx with 2 M NaOH and diluting to the mark with 50.0 mM phosphate buffer solution of pH 7.5 while stock X solutions were prepared by dilution of proper amount of this reagent with working buffer solution.

2.3. Electrode preparation

XO-based GCPE was prepared by hand mixing of 80:20 (% w/w) glassy carbon spherical powder/mineral oil with 0.3 mg XOD and 2 µL Au-np. A portion of the resulting paste was then packed firmly into the electrode cavity (3.0 mm diameter and 5.0 mm depth) of a PTFE sieve where electrical contact was established via a copper wire. Resulting electrode was put into desiccator for about 15 min for allowing the dryness of the surface. Then electrode surface was covered by a pre-soaked dialysis membrane by means of rubber o-ring in order to prevent leakage of the paste into the solution.

2.4. Procedure

Prior to its use, the surface of the composite electrode was smoothed with a weighing paper. The three electrodes were immersed into the 10 mL electrochemical cell. Chronoamperometric measurements were carried out in 50.0 mM phosphate buffer (pH 7.5) medium under the operating potential of +700 mV while the solution was stirred. The duration of each analysis was 125 s and the transient current decayed to a steady state value after 50 s in the presence of supporting electrolyte. Cyclic voltammetric experiments were conducted with same supporting electrolyte between –500 and +1250 mV; at 50 mV/s with a frequency of 50 Hz, amplitude of 20 mV and potential step of 20 mV. The electrode surface was renewed before the beginning of every measurement.

2.5. Sample application

Canned tuna fish sample (Dardanel) was purchased from a local market. The sample was divided into two parts. One part was immediately used while the other part was allowed to stand at room temperature for 15 days.

Canned tuna fish was chopped and homogenized until a fine paste was obtained after addition of 5 mL 0.5 M HClO₄. Obtained denatured samples were mechanically stirred for 10 min and then centrifuged at 4000 rpm for 5 min. The pH of the supernatant was adjusted to 7.5 with concentrated NaOH and diluted 10 times before applying standard addition method for analytical assays.

Amperometric measurements in stirred solutions were performed after transferring the corresponding analytical solution to the electrochemical cell with a potential of +700 mV versus Ag/AgCl. Determination of Hx involved two to three successive additions of samples and denatured samples which included known amount of Hx (80 µM), respectively.

3. Results and discussion

3.1. The effect of Au-np on electrochemical response of nanocomposite biosensor

Au-np has received so much attention in the field of biosensors due to its physico-chemical character [19,20]. Au-np can be defined as good biocompatible material since modification of electrode surfaces with this particle provides a suitable microenvironment similar to that of redox protein. Hence, it gives the protein molecules more freedom in orientation [21].

In order to examine its electro catalytic behavior, two biosensors; one with Au-np and other without Au-np were prepared and used for X detection. Fig. 1 displays the cyclic voltammograms at 50 mV/s of biosensors (A) without Au-np and (B) with Au-np for (a) background and (b) 10 µM X solution. Both electrodes display a defined electrochemical response upon addition of X. However, as can clearly be seen from the voltammograms, the enzymatic reactions at the XOD–Au-np–GCPE electrode begins at a substantially lower potentials (around 0.5 V), compared to XOD–GCPE (0.75 V) counterpart. Besides, the XOD–Au-

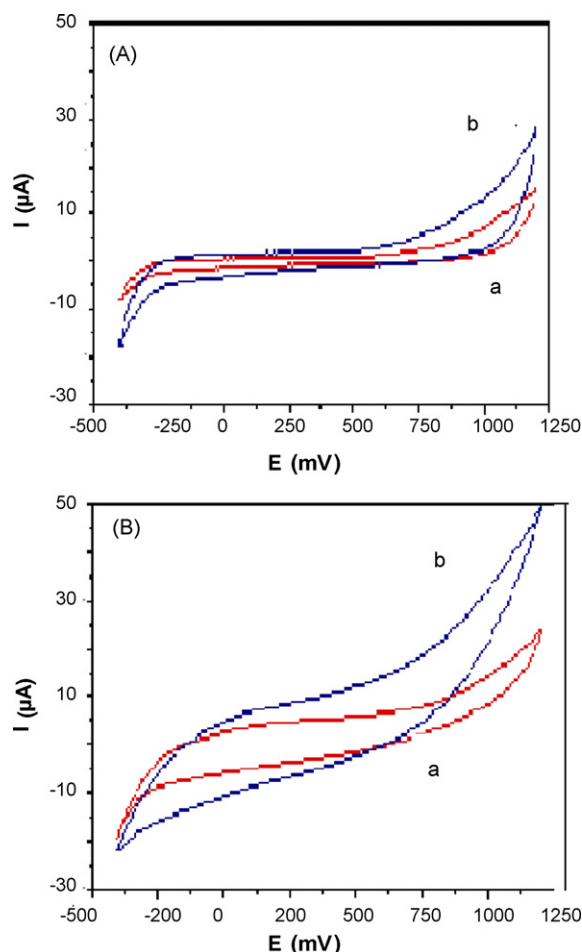


Fig. 1. Cyclic voltammograms recorded for composite biosensors (A) without Au-np and (B) with Au-np for (a) background and (b) $10 \mu\text{M}$ X solution. Conditions: supporting electrolyte, 50 mM phosphate buffer (pH 7.50) and scan rate 50 mV s^{-1} .

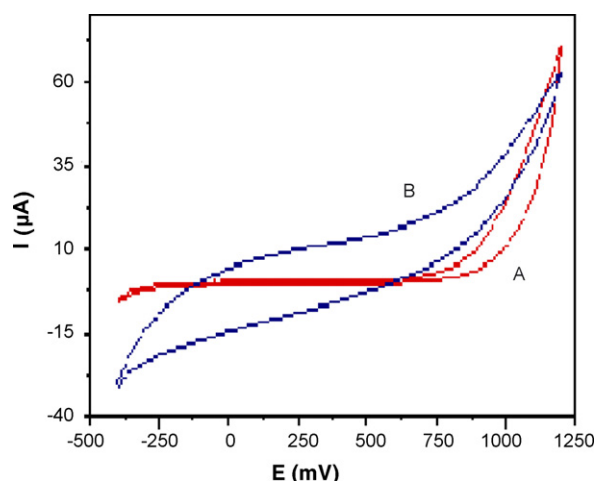


Fig. 2. Cyclic voltammograms of $10 \text{ mM H}_2\text{O}_2$ at (A) plain GCPE and (B) Au-np modified GCPE. Conditions: supporting electrolyte, 50 mM phosphate buffer (pH 7.50) and scan rate 50 mV s^{-1} .

np-GCPE nanocomposite yields also a substantially higher sensitivity over the entire potential range.

In Fig. 2, the response of (A) plain GCPE, (B) Au-np modified GCPE on the $10 \text{ mM H}_2\text{O}_2$ solution is demonstrated. As it can clearly be seen from the voltammograms, both electrodes display almost same electro catalytic behavior upon H_2O_2 solution. On the other hand, it is claimed that at lower potentials XOD enzyme reaction products could be both hydrogen peroxide and uric acid [6]. So this increase might be due to catalytic effect of Au-np to uric acid. However, it is stated that plain Au-nanoclusters have almost no catalytic activity towards uric acid [22]. Moreover to enhance the catalytic activity Au-np was used with polypyrrole film [22]. As a result, it is clear that presence of Au-np facilitates the enzymatic reaction. Au-np reduces the insulating effect of the protein shell by providing direct electron transfer through the conducting tunnels of gold nanocrystals [15]. It is claimed that the penetration of nanomeric edges of gold particles decreases the distance between the electrode and biomolecular redox sites for electron transfer [15].

3.2. The effect of applied potential

It is known that the Au-np has been used as modifier to catalyze the electrochemical reaction and direct electron transfer of some molecules like H_2O_2 [12]. In order to examine this effect optimum operating potential was searched. As shown in Fig. 3, the performance of the sensor was tested between 200 and 1000 mV with increment of 100 mV for $10 \mu\text{M}$ X solution. Although highest current value was obtained at $+900 \text{ mV}$, $+700 \text{ mV}$ was chosen as optimum potential since 77% of maximum current was attained at this potential.

Substances like ascorbic acid (AA) may present together with X and Hx in real samples and can be electrochemically oxidized at this potential value. In order to observe this effect, developed system's chronoamperometric responses at $+700 \text{ mV}$ to AA were monitored. As a result, up to $500 \mu\text{M}$ no chronoamperometric signal was obtained for AA. For this reason, it can be concluded that with developed nanobiocomposite electrode and under these working conditions, AA does not interfere to our measurements.

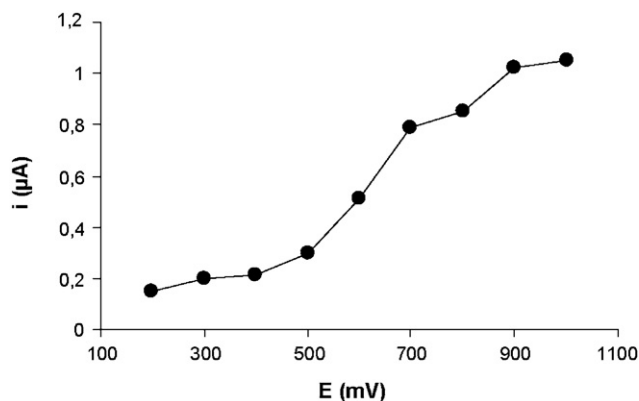


Fig. 3. Effect of working potential (200– 1000 mV) on the electrode response in phosphate buffer, 50 mM at pH 7.0, for $10 \mu\text{M}$ X with $5 \mu\text{L}$ Au-np.

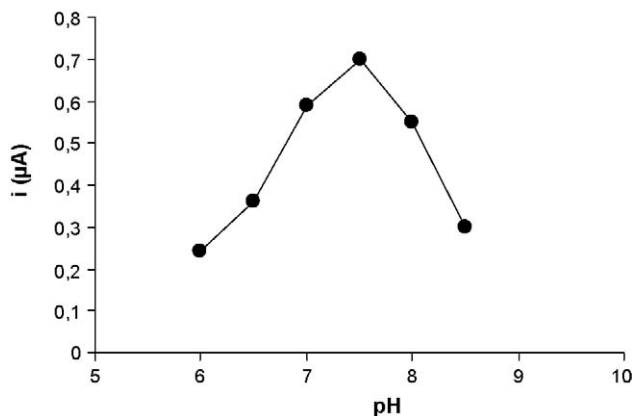


Fig. 4. Effect of pH (pH 6.0–8.5) on the biosensor response; phosphate buffer (50 mM), +700 mV, 10 μM X, with 5 μL Au-np.

3.3. The pH effect

The effect of pH on the electrode response was investigated by using phosphate buffer systems (50.0 mM) between pH 6.5 and 8.5 with an increment of 0.5 (Fig. 4). The response current of the enzyme electrode to 10 μM X increases significantly from pH 6.5 to 7.5, and then a decrease is obtained at pH values higher than 7.5. As a result pH 7.5 was chosen as optimum pH for further studies.

3.4. The influence of Au-np amount

Fig. 5 demonstrates the effect of Au-np amounts (0.5, 1, 2, 5, 10 μL) on the current response of 10 μM X. As clearly can be seen from the figure, the current increases sharply from 1 to 2 μL and then a sharp decrease is obtained for volumes greater than 2 μL. The shape of the curve is in accordance with other biosensor studies containing Au-np modification [7]. According to Liu et al. [7] this decrease can be attributed to increment of resistance and capacitance that are caused by double layer of the electrode since the active sites of the graphite decreases due to the increase in Au-np amount.

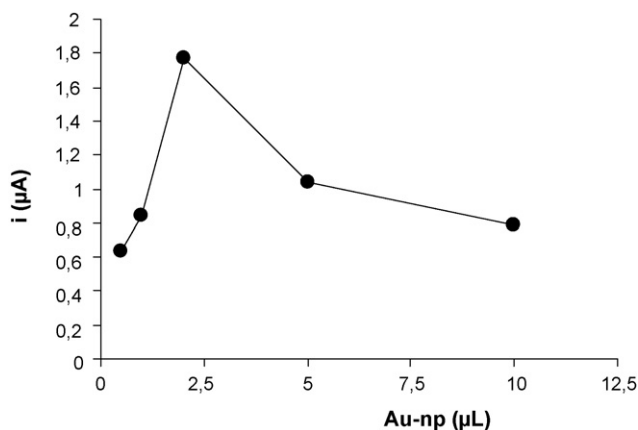


Fig. 5. The effect of Au-np amount (0.5, 1.0, 2.0, 5.0 and 10.0 μL) for 10 μM X, at pH 7.5 at +700 mV.

3.5. Surface imaging

Fig. 6 indicates the SEM images of XOD–Au-np–GCPE composite material under the optimized conditions. At (A) with 1.84 k× magnification highly packed non-porous surfaces characterized the GCPE [17] with small dots on to the spherical microparticles in the dark part. On the other hand, concerning the dimensions of the materials like glassy carbon microparticles

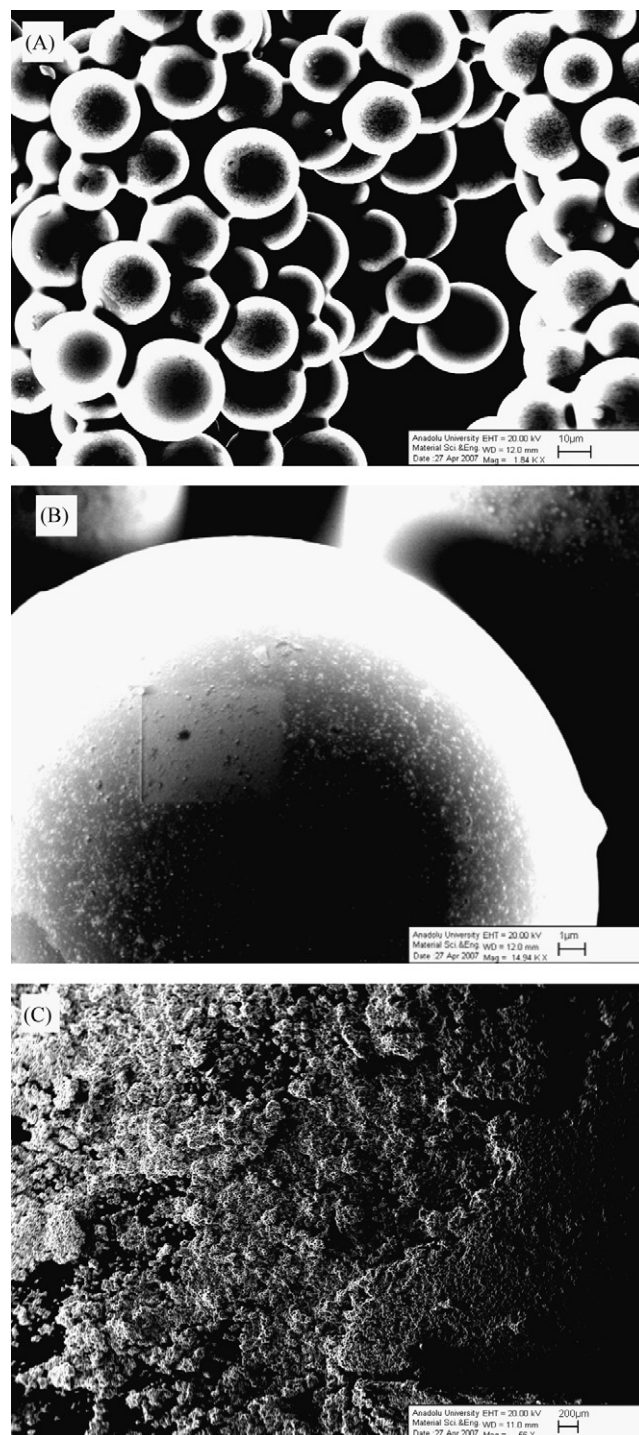


Fig. 6. The SEM images of XOD–Au-np–GCPE composite with magnification of (A) 1.84 k×, (B) 14.94 k× and (C) 36 k× accelerating voltage 20.00 kV.

20–50 μm and Au-np 10 nm, the dots at the dark part might be Au-np with XOD enzyme in it (at (B) with 14.94 k $\times$ magnification). At (C) with 36 k $\times$ magnification, a sponge-like structure of the composite can be seen.

3.6. Analytical characteristics

A linearity for the biosensor was obtained in concentration range between 5.0×10^{-7} and 1.0×10^{-5} M for X with equation of $y = 0.24x + 0.712$ and 5.0×10^{-6} to 1.50×10^{-4} M Hx, with equation of $y = 0.014x + 0.575$, respectively. R^2 values were equal to 0.99 for both reagents. For comparison, these experiments were repeated by using plain GCPE with XOD. For X, the linear range was between 2.0×10^{-6} and 1.0×10^{-5} M with equation of $y = 3.10^{-5}x + 0.0033$ while for Hx the linear range was between 20.0×10^{-6} and 80.0×10^{-6} M with an equation of $y = 0.0206x + 0.2111$. R^2 values were equal to 0.99 for X and 0.97 for Hx. As it is clearly be seen Au-np included structure possess wider linear range with better R^2 values. Also when compared with other Hx biosensors that has Au-np (modification of CPE with electrodeposited Au-np [6]) wider linear range is obtained with XOD–Au-np modified GCPE. This could be due to more efficient electrochemical oxidation of H_2O_2 and/or uric acid at this electrode potential (+700 mV). The presence of Au-np as a part of composite structure may prevent accumulation of enzymatic reaction products and hence subsequent enzyme inhibition.

The repeatability of the biosensor was tested in terms of R.S.D. values for 5 μM ($n=5$) X and 80 μM ($n=5$) Hx and calculated as 6.31 and 3.57%, respectively.

Storage stability was also checked and the enzyme electrode was kept at +4 °C between the measurements. Only 18% of activity was diminished after 1 week.

3.7. Sample application

Hx level in meat and marine products are important as a food quality control index. Canned tuna fish samples which are divided two parts (one part was used as a fresh sample and the other part that was labeled as decomposed sample was waited at room temperature for 15 days before its usage) were used for Hx detection. Standard addition assay was also applied to evaluate the sample matrix effect.

As already mentioned in Section 2, perchloric acid was used for deproteinisation of samples by providing denaturation of proteins. After centrifugation, clear supernatant solution was obtained. Standard HX solution was added to one part of this supernatant solution to obtain final concentration of 80 μM while other part used without any Hx standard solution inside. Both solutions were subjected to standard addition in other words, samples with and without standard HX were used as stock substrate solutions and added to the reaction cell after equilibration. Hx amount in samples was calculated from calibration curve.

The recovery values for fresh and decomposed samples were calculated as 102.5 and 106%, respectively. The samples that did not contain standard Hx solution did not give any signal.

Higher recovery value for decomposed sample can be attributed to interferences of other compounds which are caused by the microbial contamination. On the other hand, the recovery of the fresh sample was close to 100%. Since the obtained signals from spiked samples were very close to the standard solutions, it can be concluded that the nature of sample does not affect the measurement.

3.8. Comparison of performance of biosensor with other Au-np contained HX biosensors

Au-np modified electrode surfaces can be prepared in different ways: (1) by binding gold nanoparticles with functional groups of self-assembled monolayers (SAMs); (2) by direct deposition of nanoparticles onto the bulk electrode surface; (3) by incorporating colloidal gold into the electrode by mixing the gold with the other components in the composite electrode matrix. Biosensors can then be constructed by immobilizing the biomolecules via adsorption onto the nanoparticles, by cross-linking them with bifunctional agents such as glutaraldehyde, or by mixing them with the other components of composite electrodes [23].

Pingarron and co-workers [6] compared the response of some of other Hx biosensors in the presence/absence of Au-np in structure and found that highest current value (87.4 nA for 5 μM Hx) was obtained with glutaraldehyde (GA)–BSA cross-linked XOD electrodeposited Au colloidal CPE. When using the same enzyme amount and under the same operating potential (+600 mV), the XOD–Au-np–GCP electrode exhibited 91 nA ($n=3$) for same amount of Hx. The slightly higher current value can be attributed to the fact that the immobilized enzyme retained higher bioactivity into the composite electrode material, giving rise to fast, stable and sensitive responses to the concerning substrate. Being more practical and providing effective signals, makes developed biosensor a robust tool for Hx detection.

4. Conclusion

XOD–Au-np–GCPE provides easy to prepare and useful biosensor for X and Hx detection. It can simply be prepared by mixing proper amount of XOD, Au-np and GC microparticles and mineral oil. From the results, it can be concluded that especially for Hx detection, more practical and efficient biosensor has been obtained. This can be explained by the composite structure that contains GC microparticles with Au-np. Au-np catalyzes the enzymatic reaction by allowing direct electron transfer between the electrode and enzyme active center by means of the conducting tunnels of gold nanocrystals [16]. On the other hand, the combination of these two materials into a composite structure rather than usage of Au-np as an immobilizing reagent, may cause the immobilized enzyme to have higher bioactivity that results fast, stable and sensitive responses to the concerning substrate.

Congratulations

To Dr. Wang,

“Anyone who stops learning is old, whether at twenty or eighty. Anyone who keeps learning stays young. The greatest thing in life is to keep your mind young. . .” Henry Ford.

Happy Birthday! I know that you will always stay young...



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References

- [1] J.-M. Zen, Y.-Y. Lai, H.-H. Yang, A. Senthil Kumar, *Sens. Actuators B* 84 (2002) 237.
- [2] L.D. Mello, L.T. Kubota, *Food Chem.* 77 (2002) 237.
- [3] V. Venugopal, *Biosens. Bioelectron.* 17 (2002) 147.
- [4] J. Pei, X.-Y. Li, *Anal. Chim. Acta* 414 (2000) 205.
- [5] A.K. Sarker, H. Ukeda, D. Kawana, M. Sawamura, *Anal. Sci.* 15 (1999) 1141.
- [6] L. Agüi, J. Manso, P. Yáñez-Sedeno, J.M. Pingarrón, *Sens. Actuators B* 113 (2006) 272.
- [7] S. Liu, J. Yu, H. Ju, *J. Electroanal. Chem.* 540 (2003) 61.
- [8] A. Doron, E. Katz, I. Willner, *Langmuir* 11 (1995) 1313.
- [9] K.R. Brown, A.P. Fox, M.J. Natan, *J. Am. Chem. Soc.* 118 (1996) 1154.
- [10] S. Liu, L. Wang, F. Zhao, *J. Electroanal. Chem.* 602 (2007) 55–60.
- [11] H.X. Ju, S.Q. Liu, B. Ge, F. Lisdat, F.W. Scheller, *Electroanalysis* 14 (2002) 141.
- [12] Y. Xiao, H.X. Ju, H.Y. Chen, *Anal. Biochem.* 278 (2000) 22.
- [13] S.Q. Liu, H.X. Ju, *Anal. Biochem.* 307 (2002) 110.
- [14] H.Y. Gu, A.M. Yu, H.Y. Chen, *J. Electroanal. Chem.* 516 (2001) 119.
- [15] M.L. Mena, P. Yáñez-Sedeno, J.M. Pingarrón, *Anal. Biochem.* 336 (2005) 20.
- [16] J. Manso, L. Agüi, P. Yáñez-Sedeno, J.M. Pingarrón, *Anal. Lett.* 37 (2004) 887.
- [17] J. Wang, Ü. Anik-Kirgoz, J.W. Mo, J. Lu, A.N. Kawde, A. Muck, *Electrochem. Commun.* 3 (2001) 3600.
- [18] Ü. Anik-Kirgoz, S. Timur, J. Wang, A. Telefoncu, *Electrochem. Commun.* 6 (2004) 913.
- [19] D. Li, J.H. Li, *Surf. Sci.* 522 (2003) 105.
- [20] Y. Xiao, H.X. Ju, H.Y. Chen, *Anal. Chim. Acta* 391 (1999) 73.
- [21] B.-Y. Wu, S.-H. Hou, F. Yin, J. Li, Z.-X. Zhao, J.-D. Huang, Q. Chen, *Biosens. Bioelectron.* 22 (2007) 838.
- [22] J. Li, X.Q. Lin, *Anal. Chim. Acta* 596 (2007) 222.
- [23] P. Yáñez-Sedeno, J.M. Pingarroñ, *Anal. Bioanal. Chem.* 382 (2005) 884.

Electrochemical determination of homocysteine at a gold nanoparticle-modified electrode

L. Agüí, C. Peña-Farfal, P. Yáñez-Sedeño, J.M. Pingarrón*

Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040 Madrid, Spain

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Abstract

The construction of a colloidal gold-cysteamine-carbon paste electrode, Au_{coll}-Cyst-CPE, for the electrochemical determination of homocysteine is reported. The improved voltammetric behaviour of homocysteine at Au_{coll}-Cyst-CPE with respect to that observed at a gold disk electrode is attributed to an enhanced electron transfer kinetics as a consequence of the array distribution of gold nanoparticles immobilized onto the Cyst SAM. Cyclic voltammetry of homocysteine showed an adsorption-controlled current for scan rates between 500 and 5000 mV s⁻¹. The hydrodynamic voltammogram constructed for homocysteine allowed the selection of a potential value of +600 mV, where the background current is negligible, for the amperometric detection of the analyte at the Au_{coll}-Cyst-CPE. Using a flow rate of 0.8 ml min⁻¹, the R.S.D. value for *i*_p after 25 repetitive injections of homocysteine was of 4.3%, and one single electrode could be used for more than 15 days without any treatment or regeneration procedure of the modified electrode surface. An HPLC method for the separation and quantification of homocysteine and related thiols, using amperometric detection at the modified electrode has been developed. A mobile phase consisting of 2:98% (v/v) acetonitrile:0.05 mol l⁻¹ buffer solution of pH 2.0, and a detection potential of +0.80 V were selected. Separation with baseline resolution and retention times of 3.00, 3.60, 4.52, 5.71 and 7.79 min were obtained for cysteine, homocysteine, glutathione, penicillamine and *N*-acetyl-cysteine, respectively. Calibration graphs were constructed for all the separated compounds. Detection limits ranged between 20 nM for cysteine and 120 nM for penicillamine, with a value for homocysteine of 30 nM. These values compare advantageously with those achieved with previously reported HPLC methods using electrochemical, UV, fluorescence and MS detection modes. The developed method was applied to the determination of cysteine and homocysteine serum samples with good results.

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1. Introduction

Homocysteine (2-amino-4-mercaptobutyric acid; hCys) is an important thiol group containing amino acid. It is produced as an intermediate in the synthesis of cysteine from parent methionine ingested in diet. Normal levels of hCys in plasma from healthy individuals are between 5 and 16 nmol ml⁻¹ [1]. Higher concentrations of hCys giving rise to hyperhomocysteinemia (with hCys up to 100 nmol ml⁻¹) or homocystinuria (around 500 nmol ml⁻¹) are associated to different diseases [2]. The hCys levels in plasma are better correlated with the risk of atherosclerosis than those of cholesterol [3]. Genetic defects or,

more frequently, an insufficient intake of vitamins B₆, B₁₂ and folic acid, which are necessary for the metabolism of hCys, are responsible of an abnormal increase of the hCys concentration [4].

Different forms of homocysteine can be found in human plasma. A small amount is in its reduced form, whereas the highest concentration corresponds to the oxidized form (disulphide). Moreover, approximately 70% of total homocysteine is bound to proteins in the form of conjugates, while the remainder 30% forms mixed disulphides with other thiols, mainly Cys-hCys [5]. Total homocysteine, i.e. the sum of free hCys and bound hCys is the clinical parameter of interest, which is needed to be determined.

Chromatographic methods are commonly used for the determination of total homocysteine in biological samples. Gas chromatography has been very scarcely used because of the high polarity of hCys and related compounds, which makes

* Corresponding author.

E-mail address: pingarro@quim.ucm.es (J.M. Pingarrón).

necessary a previous derivatization to increase volatility [6]. Liquid chromatography with UV [7,8] or fluorescence [9,10] detection has been frequently used, although derivatization treatments in order to introduce chromophore or fluorophore groups are required. LC–MS provides a useful analytical tool for the identification and quantification of these compounds [11].

HPLC with electrochemical detection constitutes an important tool for the determination of thiols and disulphides [12] with the main advantage that derivatization is not necessary. However, large overpotentials are required for the electrooxidation reaction of homocysteine at conventional electrodes [13]. Electrochemistry of thiol compounds revealed a slow behaviour at solid electrodes [14] with an overall electrochemical process, similar to that occurring in biological systems, which implies the interconversion of the redox couple RSH/RSSR [15]. Adsorption phenomena and the effect of electrode surface oxides complicate the electrochemical oxidation of thiols at these electrodes [16,17]. Chemically modified electrodes have been used to overcome such inconvenients [18,19]. Moreover, pulsed electrochemical detection at gold electrodes has been also used for the cleaning and activation of the electrode surface in the determination of thiols [20]. Recently, the electrochemical detection of homocysteine at a carbon nanotube-modified electrode [21], and at a platinum/poly(methyl violet) modified electrode [22] have been reported.

Gold nanoparticles have demonstrated their usefulness for the preparation of electrochemical sensors giving rise to improved responses from biologically important compounds with respect to those observed at conventional electrode surfaces [23]. Recently, we demonstrated that carbon paste electrodes (CPEs) modified with cysteamine (Cyst) constitute excellent electrode surfaces for the preparation of gold nanoparticle-modified electrodes [24]. These modified electrodes exhibited good analytical characteristics towards analytes containing sulfur atoms, such as methionine [25].

In this article, a colloidal gold-cysteamine-carbon paste electrode, Au_{coll}-Cyst-CPEs, was constructed for the improved electrochemical determination of homocysteine. The capability of this modified electrode to be used as an amperometric detector in the HPLC determination of thiol derivatives is demonstrated.

2. Experimental

2.1. Apparatus and electrodes

Voltammetric measurements were carried out with a BAS (West Lafayette, IN, USA) 100B potentiostat provided with a BAS C2 EF-1080 cell stand. Colloidal gold-cysteamine-carbon paste electrodes were used as working electrodes. Carbon paste electrodes were prepared by mixing 0.25 g graphite powder (ultra “F” purity) with 90 μ l mineral oil (Sigma) and stirring for 15–20 min. The resulting paste was packed into the well of a cylindrical Teflon support (3 mm in diameter, geometric area 0.0707 cm²) provided with a stainless steel screw as the electric contact. A Metrohm 6.1204.020 Au disk electrode (3 mm in diameter) was also used for comparison purposes. This electrode was polished with 3 μ m diameter diamond powder before use.

The reference electrode was a BAS MF 2063 Ag/AgCl electrode and a BAS MW-1032 Pt wire was used as the auxiliary electrode. BAS VC-2 10 ml electrochemical cells were also used.

Chromatographic and flow injection experiments were performed using a Knauer 1000 Smartline pump provided with a Knauer 5000 Smartline Manager stand, and a Knauer A 1357 injection valve provided with a 50 μ l coil. An Ultrabase C₁₈ (150 mm $\times$ 4.6 mm i.d., 5 μ m particle size) (Scharlab) chromatographic column was used. The flow-cell was a Metrohm EA-1096 wall-jet cell provided with a 3 M Ag/AgCl/KCl reference electrode and a gold counter electrode. The potential values applied were controlled by means of a BAS Epsilon Multichannel detector, and the Chromgraph 1.0.01 software (Liquid Chromatography Control Software) from BAS was used to record data.

2.2. Reagents and solutions

Stock 1.0×10^{-2} M aqueous solutions of D-homocysteine (Fluka, 99%), L-cysteine, N-acetyl-L-cysteine, L-penicillamine and L-glutathione (Sigma, 99%) were used. For the preparation of the modified electrodes, aqueous 1% HAuCl₄·3H₂O (Sigma, >49.0% as Au), 1% sodium citrate (Panreac, 98%) and 0.40 mol l⁻¹ cysteamine hydrochloride (2-aminoethanethiol) (Aldrich, 98%) were employed. A 0.10 mol l⁻¹ H₂PO₄⁻/HPO₄²⁻ (Scharlab, 99%) buffer solution of pH 7.0 and a Britton–Robinson buffer solution containing each component acid at 0.2 mol l⁻¹, whose pH values were adjusted by suitable addition of a 2 mol l⁻¹ NaOH solution, were used. All solvents and chemicals were of analytical reagent grade. The water used was obtained from a Millipore Milli-Q system.

2.3. Procedures

2.3.1. Preparation of Au_{coll}-Cyst-CPEs

Firstly, colloidal gold nanoparticles (24 nm in diameter) was prepared as described previously [24], by adding 0.66 ml of sodium citrate solution to a boiling 50 ml solution of 0.01% HAuCl₄. Then, colloidal gold-cysteamine-modified carbon paste electrodes were prepared as follows [24]. The carbon paste electrode (CPE) was smoothed with a clean paper card until a shiny surface was obtained. Next, it was immersed in a 0.10 mol l⁻¹ Cyst solution for 2 h at room temperature without stirring, and washed with distilled water to remove the unbond thiol molecules. Finally, the Cyst-CPE was immersed in the colloidal gold suspension for 4 h at 4 °C in the darkness. The modified electrode was stored in the refrigerator until use.

2.3.2. Determination of homocysteine in serum samples

Lyophilized serum (Accutrol from Sigma) was reconstituted with 5.0 ml Milli-Q water. After complete dissolution, a 500 μ l aliquot was treated with 500 μ l of a 10% (w/v) sodium borohydride solution by incubation at 50 °C during 30 min. Then, 1250 μ l of a 1:4 HClO₄–water solution was added and the mixture was homogenized by stirring and centrifuged at 3000 rpm for 10 min. Next, the liquid was filtered through a 22 μ m Nylon membrane. The determination of homocysteine was carried

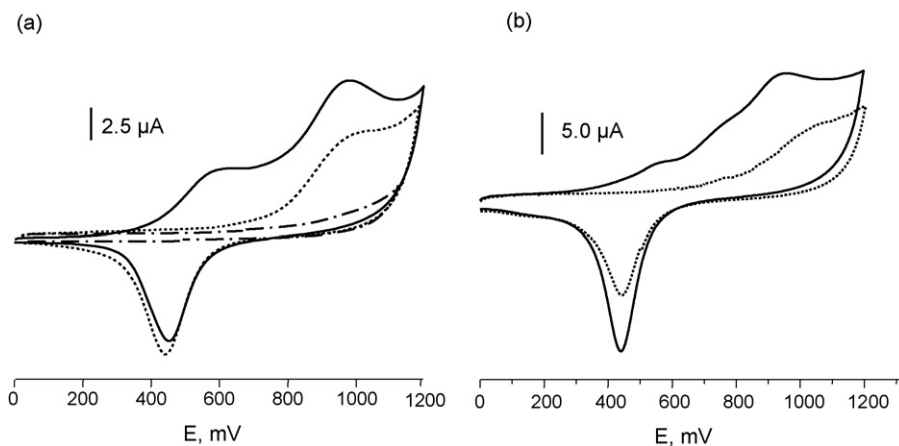


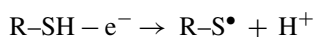
Fig. 1. Cyclic voltammograms from $1.0 \times 10^{-4} \text{ mol l}^{-1}$ homocysteine in 0.1 mol l^{-1} phosphate buffer solution of pH 7.0 at: (a) (---, 1) Au_{coll} -Cyst-CPE; (- · -, 2) Cyst-CPE and bare CPE, and (b) (—, 1) a gold disk electrode; (· · ·, 3) background voltammograms; $\nu = 50 \text{ mV s}^{-1}$.

out by injection of $50 \mu\text{l}$ aliquots of the filtered solution into the HPLC system using the Au_{coll} -Cyst-CPE as amperometric detector.

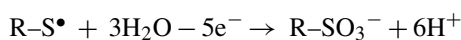
3. Results and discussion

3.1. Voltammetry of homocysteine at Au_{coll} -Cyst-CPE

Cyclic voltammograms from a $1.0 \times 10^{-4} \text{ mol l}^{-1}$ homocysteine solution recorded at a Au_{coll} -Cyst-CPE and at a Cyst-CPE and bare CPE electrodes are displayed in Fig. 1. As it can be seen, two well-defined oxidation peaks at +600 and +950 mV appeared at the gold nanoparticle-modified electrode. However, no peaks were observed at both Cyst-CPE and bare CPE. The voltammetric signal obtained at +950 mV in the background voltammogram corresponds to well-known formation of gold oxides at these positive potential values. Moreover, Fig. 1 also shows the cyclic voltammogram obtained at a conventional gold disk electrode. As can be observed, although similar qualitative voltammetric behaviour of homocysteine was found, the voltammogram is much poorer defined appearing as a broad anodic band. These results agree with those reported for other thiol molecules such as cysteine at Au electrodes [20] and at a Au_{coll} -Cyst-CPE [24]. Accordingly, the oxidation peak at +600 mV should correspond to the electrochemical reaction [15]:



The following oxidation occurs concomitantly with the formation of the surface gold oxide. In this step, the transfer of oxygen to the adsorbed thiocompound is enhanced by the labile surface oxides thus occurring an oxide-catalyzed process which probably produces a sulfonic acid [15]:



Obviously, the cathodic peak observed at approximately +450 mV is due to the reduction of the gold oxides formed during the anodic scan.

The improved voltammetric behaviour of homocysteine at the Au_{coll} -Cyst-CPE can be attributed to the enhanced electron

transfer kinetics as a consequence of the array distribution of the gold nanoparticles immobilized onto the Cyst SAM.

The effect of pH on the oxidation peak current and peak potential of homocysteine revealed that only a broad single oxidation peak appeared at pH values between 2.0 and 6.0. From pH 6, voltammograms showed the two peaks displayed in Fig. 1. In alkaline media, the oxidation current decreased when pH increased, and the peaks practically disappeared at pH 11. The E_p value (which corresponds to the less positive peak when two peaks appeared in the voltammograms) varied with pH as it is displayed in Fig. 2, with a linear dependence ($r = 0.996$; $n = 7$) between pH 3 and 9, showing a slope value of -76 mV . This value, higher than the theoretical one for the exchange of the same number of electrons and protons (-59 mV) is probably due to the adsorption of homocysteine at the electrode surface. The slope value changed significantly at pH values of 2.9 and 9, which are near to homocysteine $\text{p}K_a$ values: $\text{p}K_{a1} = 2.22$ and $\text{p}K_{a2} = 8.87$ [13], for carboxylic and thiol groups, respectively.

The influence of the potential scan rate on the voltammetric response of $1.0 \times 10^{-4} \text{ mol l}^{-1}$ homocysteine was evaluated

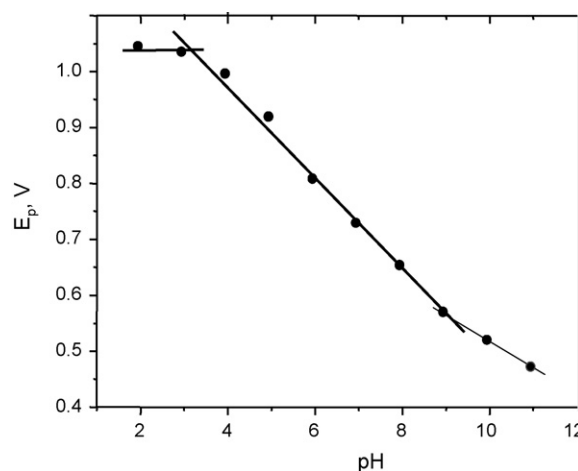


Fig. 2. Influence of pH on peak potential values obtained from cyclic voltammograms of $1.0 \times 10^{-4} \text{ mol l}^{-1}$ homocysteine at a Au_{coll} -Cyst-CPE in 0.1 mol l^{-1} Britton–Robinson buffer; $\nu = 50 \text{ mV s}^{-1}$.

over the $2\text{--}5000\text{ mV s}^{-1}$ range in a 0.1 mol l^{-1} phosphate buffer of pH 7.0 as background solution. The results obtained for the less positive oxidation peak showed a shift on E_p towards more positive values as the scan rate increased as corresponds to an irreversible electrochemical behaviour. Peak current increased with the potential scan rate, giving a linear i_p versus $v^{1/2}$ plot ($r=0.995$; $n=9$) for the lower scan rates used ($2\text{--}250\text{ mV s}^{-1}$). Conversely, between 500 and 5000 mV s^{-1} , i_p varied linearly with v ($r=0.997$; $n=5$), a linear $\log i_p$ versus $\log v$ plot with a slope value practically equal to one being obtained. This behaviour confirmed the existence of an adsorption process in the electrochemical oxidation of homocysteine at the Au_{coll} -Cyst-CPE.

3.2. Amperometric detection of homocysteine at Au_{coll} -Cyst-CPE

Fig. 3 shows the hydrodynamic voltammogram constructed by measuring homocysteine peak current at different applied potentials, after injection of $50\text{ }\mu\text{l}$ aliquots of a $5.0 \times 10^{-5}\text{ mol l}^{-1}$ homocysteine solution into the carrier consisting of 0.1 mol l^{-1} phosphate buffer of pH 7.0. As can be seen, a significant different current for homocysteine with respect to the background current was found from $+200\text{ mV}$. Moreover, the background signal observed for potential values more positive than $+600\text{ mV}$ suggests the contribution of gold oxidation processes to the current measured at these positive potentials. Therefore, 600 mV , where the background current was negligible, was selected as the potential value to be applied for the amperometric detection of homocysteine at the Au_{coll} -Cyst-CPE.

The peak current increased and the peak width decreased when the flow rate increased over the $0.3\text{--}1.5\text{ ml min}^{-1}$ range. However, the repeatability of the flow injection responses was poorer for the higher flow rates used (i.e. R.S.D. for i_p was 11% ($n=25$) when a flow rate of 1.5 ml min^{-1} was used), which is probably due to a lower stability of the electrode modifier layer.

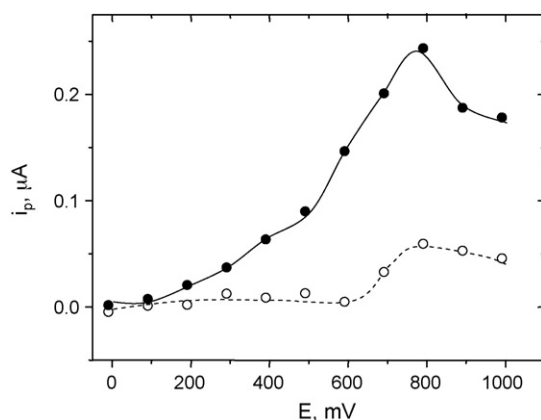


Fig. 3. Hydrodynamic voltammogram obtained by flow injection with amperometric detection at a Au_{coll} -Cyst-CPE after injection of $50\text{ }\mu\text{l}$ aliquots of $5.0 \times 10^{-5}\text{ mol l}^{-1}$ homocysteine into a carrier consisting of a 0.1 mol l^{-1} phosphate buffer solution of pH 7.0; (---) background voltammogram; $q=0.8\text{ ml min}^{-1}$.

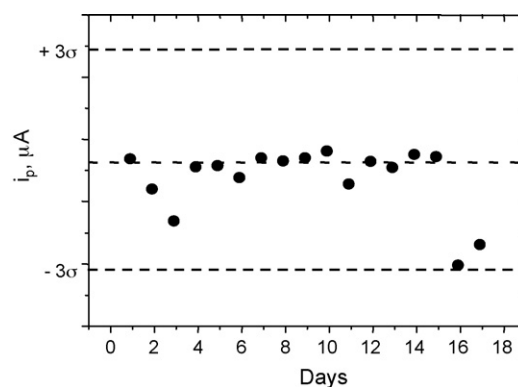


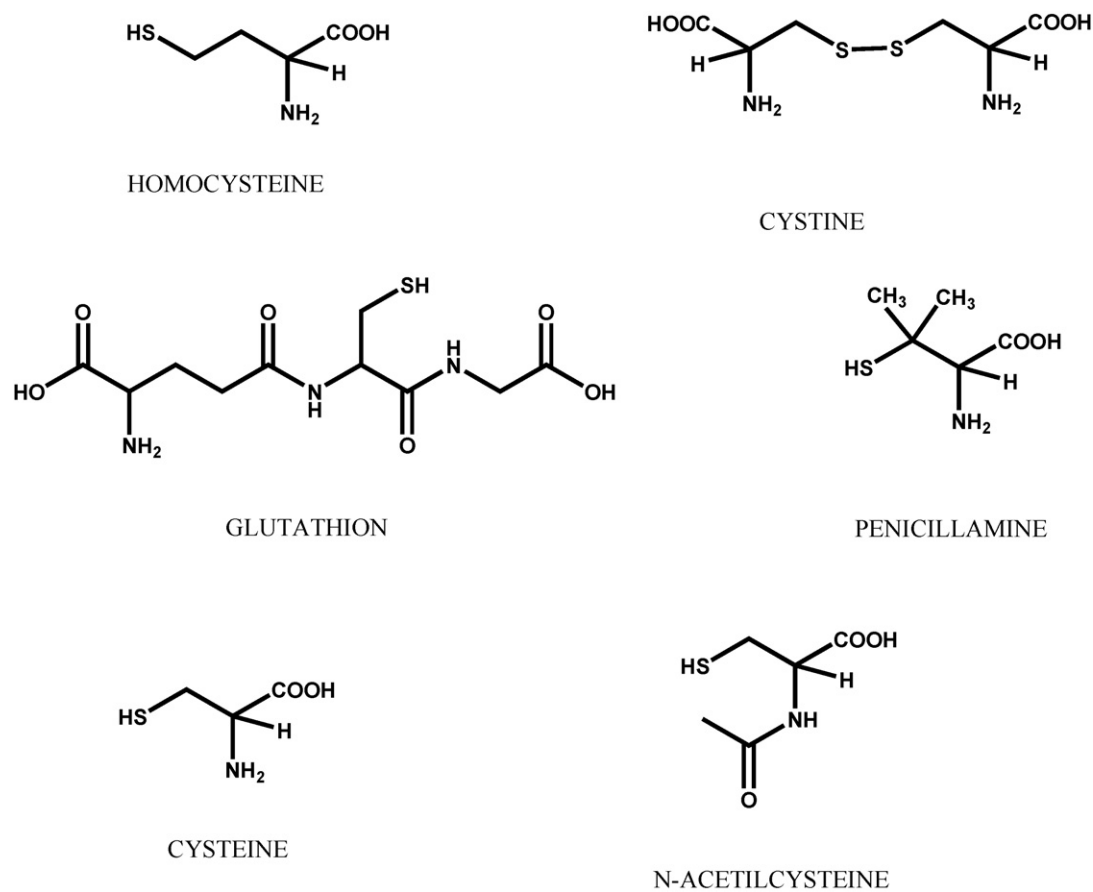
Fig. 4. Control chart constructed for a Au_{coll} -Cyst-CPE under flow injection conditions. Data show the mean values of three amperometric measurements from different $5.0 \times 10^{-5}\text{ mol l}^{-1}$ homocysteine solutions. $E_{\text{app}} = +0.6\text{ V}$.

Therefore, as a compromise between sensitivity and precision, a flow rate of 0.8 ml/min was selected for further work. Using this flow rate, the R.S.D. value for i_p after 25 repetitive injections of homocysteine was of 4.3%.

The operational stability of one single Au_{coll} -Cyst-CPE under flow injection conditions was checked by performing daily three measurements from a $5.0 \times 10^{-5}\text{ mol l}^{-1}$ homocysteine solution. Fig. 4 displays the control chart constructed for this purpose, where the mean value of 10 successive measurements was taken as the central value, and the upper and lower limits of control were set at $\pm 3 \times$ the standard deviation of this target value. As it can be observed, the mean values remained inside the control limits for more than 15 days without any treatment or regeneration procedure of the modified electrode surface. During this period of time, the electrode was stored under dry conditions at 4°C when it was not used.

Since acetonitrile and methanol have been used as mobile phase components for the chromatographic determination of homocysteine and other thiols [7,9], the effect of both solvents on the amperometric response of homocysteine was tested. As expected, taking into account the decrease in the dielectric constant and conductivity of solutions containing organic solvents, the peak current decreased as the percentage of organic solvent in the working solution increased over the 0.25–2% (v/v) checked range. A 70% of the peak current obtained in aqueous medium remained for the highest solvent percentage tested in both cases. Moreover, background current and peak-to-peak noise were similar for both solvents, although acetonitrile caused a lower long-term damage of the electrode surface than that observed using methanol.

Different compounds related with homocysteine: glutathione, cysteine, cystine, *N*-acetylcysteine and a drug used for cystinuria disease, penicillamine (see Scheme 1), were amperometrically tested, under the experimental conditions mentioned above, at the Au_{coll} -Cyst-CPE. With the exception of cystine, a disulphide that probably requires the application of more positive potentials to be oxidized, all the other compounds produced oxidation amperometric signals.



Scheme 1.

3.3. HPLC with amperometric detection at Au_{coll} -Cyst-CPE

Considering the above-mentioned results, we developed an HPLC method for the separation and quantification of homocysteine and related thiols, using amperometric detection at the modified electrode. The method was implemented using isocratic elution and a C_{18} reversed-phase column [7,8]. The composition of the mobile phase was optimized using homocysteine, cysteine and glutathione as target compounds. Firstly, the influence of the pH value on the separation of such species was evaluated using 2:98% (v/v) acetonitrile:0.1 mol l⁻¹ phosphate buffer solutions prepared at different pHs in the 2.0–7.0 range. The resolution increased as pH value decreased, and at pH 2.0, the separation was almost complete. Consequently, this pH value was selected for the chromatographic determination of homocysteine and related compounds. Since this pH value is rather different than that employed for voltammetric and flow injection with amperometric detection studies, a further optimization of the potential value used for the amperometric detection under the HPLC working conditions was needed. Fig. 5 shows both the cyclic and the hydrodynamic voltammograms of homocysteine at pH 2.0. As indicated in Section 3.1, a single oxidation peak appeared in the CV with a E_p value of 1040 mV. The hydrodynamic voltammogram showed a well-defined current “plateau” from 800 V. Moreover, a stable background current was observed. Consequently, +0.80 V was selected to carry out

the chromatographic detection. The amperometric responses, as well as peak resolution were not affected by the phosphate buffer concentration in the 0.01–0.1 mol l⁻¹ range, and a 0.05 mol l⁻¹ concentration was used in the mobile phase to carry out the separation.

Fig. 6 shows the chromatogram obtained for a solution containing 3.0×10^{-5} mol l⁻¹ of each homocysteine, cysteine, glutathione, *N*-acetylcysteine and penicillamine. As can be observed, a good separation was achieved, with baseline resolution and adequate retention times, these being 3.00, 3.60, 4.52, 5.71 and 7.79 min for cysteine, homocysteine, glutathione, penicillamine and *N*-acetylcysteine, respectively.

Calibration graphs were constructed for all the separated compounds by measuring the respective peak areas. The analytical characteristics of these calibration graphs are summarized in Table 1. Detection limits were calculated as the analyte concentration that produced a signal-to-noise ratio of three. The values obtained ranged between 20 nM for cysteine and 120 nM for penicillamine, with a detection limit for homocysteine of 30 nM. These analytical characteristics are compared with those provided by some other HPLC methods using electrochemical (ED), ultraviolet (UV) fluorescence (FL) and mass spectrometric (MS) detection modes (Table 2). As it can be seen, the detection limits are generally better than those achieved by the previously reported methods, even using MS detection [26]. In particular, and comparing with HPLC-ED

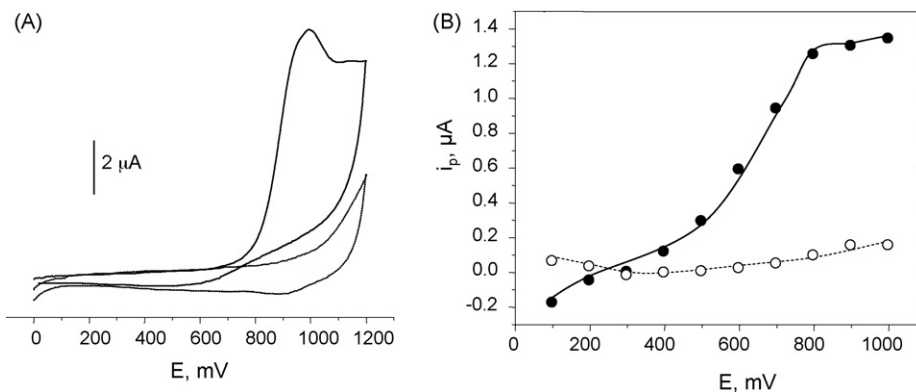


Fig. 5. (A) Cyclic voltammogram from $1.0 \times 10^{-4} \text{ mol l}^{-1}$ homocysteine in 0.1 mol l^{-1} phosphate buffer solution of pH 2. (B) Hydrodynamic voltammogram obtained by injection of $50 \mu\text{l}$ aliquots of $1.0 \times 10^{-4} \text{ mol l}^{-1}$ homocysteine into a carrier consisting of a 0.1 mol l^{-1} phosphate buffer solution of pH 2.0; (---) background voltammograms.

Table 1

Analytical characteristics of the calibration graphs obtained for homocysteine and related thiols by HPLC with amperometric detection at the $\text{Au}_{\text{coll}}\text{-Cyst-CPE}$

Species	Linear range (μM)	Slope ($\times 10^{-4} \mu\text{C M}^{-1}$)	Intercept ($\times 10^3 \mu\text{C}$)	r	R.S.D. (%) ^a	Detection limit (μM) ^b
Cysteine	0.1–5	7.7 ± 0.1	3 ± 1	0.9995	4.3	0.02
Homocysteine	0.1–5	6.1 ± 0.3	11 ± 5	0.992	5.0	0.03
Glutathion	0.1–2.5	4.2 ± 0.3	2 ± 1	0.990	5.0	0.09
Penicillamine	0.5–10	4.5 ± 0.1	6 ± 2	0.996	4.9	0.12
<i>N</i> -Acetylcysteamine	0.1–1	2.0 ± 0.1	1.4 ± 0.6	0.990	5.0	0.03

$V_{\text{inj}} = 50 \mu\text{l}$; $E_{\text{det}} = +800 \text{ mV}$. Points included in the calibration graph are 15 for cysteine, homocysteine and penicillamine, 13 for glutathion and 10 for *N*-acetylcysteamine.

^a For peak areas ($n = 10$) at a concentration $1.0 \times 10^{-6} \text{ M}$.

^b $S/N = 3$.

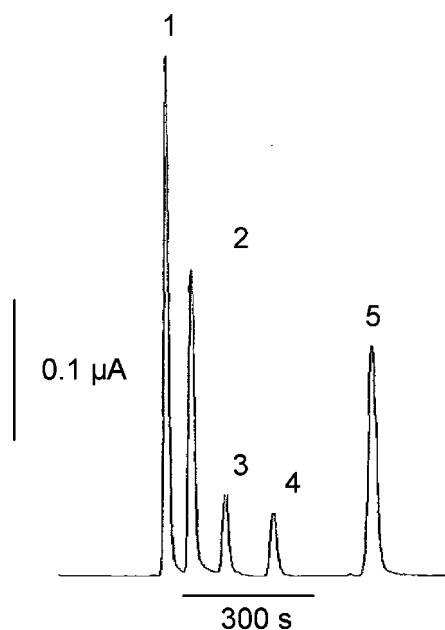


Fig. 6. Chromatogram obtained from a standard solution containing $3.0 \times 10^{-5} \text{ mol l}^{-1}$ each (1) cysteine; (2) homocysteine; (3) glutathion; (4) penicillamine and (5) *N*-acetylcysteine with amperometric detection at a $\text{Au}_{\text{coll}}\text{-Cyst-CPE}$. Mobile phase, 2% (v/v) acetonitrile: 0.05 mol l^{-1} phosphate buffer of pH 2.0; $V_{\text{inj}} = 50 \mu\text{l}$; $E_{\text{app}} = +0.8 \text{ V}$; $q = 0.8 \text{ ml min}^{-1}$.

methods, the detection limit for homocysteine is improved with respect to coulometric detection and pulsed electrochemical detection [27,20] and is similar to that obtained with a series dual detection using Au/Hg electrodes [12]. Particularly interesting is the advantageous comparison with the method using a Pt nanoparticle/poly(methyl violet) modified electrode [22].

3.4. Determination of homocysteine in serum samples

The developed HPLC method with amperometric detection at the $\text{Au}_{\text{coll}}\text{-Cyst-CPE}$ was applied to the determination of homocysteine in a serum sample. The sample treatment procedure described in Section 2.3.2 was based on the reduction of disulfides and release of the protein-bound homocysteine [22]. As an example, Fig. 7 shows one of the chromatograms recorded which exhibited peaks for cysteine and homocysteine together with a very intense peak at a higher retention time that corresponded to uric acid. Calibration plots obtained by standard additions for both thiols revealed the existence of a matrix effect, giving rise to slope values two and three times lower, respectively than those given in Table 1. The mean concentration values from six replicates were 1.5 ± 0.2 and $4.1 \pm 0.3 \mu\text{M}$ for cysteine and homocysteine, respectively. In order to evaluate the accuracy of the method, samples spiked with a known concentration, $4.0 \times 10^{-6} \text{ M}$ of each species were prepared. Recovery studies yielded a

Table 2
A comparison of the analytical characteristics of different electrochemical tyrosinase biosensor

Technique	Analytes	Sample	Method	Linear range (μM)	LOD ^a (μM)	Reference
HPLC-UV	Cys, hCys, Cys Gly	Blood, urine, plasma	Reduction with TCEP; SPE purification; separation with C8 + Spherisorb ODS2; elution with 2.5% AcCN–1% HOAc	2.5–10	0.1	[7]
HPLC-UV	Cys, hCys Cys Gly, GSH	Plasma	C CMTQ treatment; deproteinization; gradient elution with 0.05 M TCA–LiOH pH 3.2–AcCN	0.5–50	0.1	[8]
HPLC-FL	hCys	Plasma	Reduction with DTT; derivatization with mBBR; gradient separation with 30 mM TBA–20% MeOH up to 100% MeOH	3.9–62.5	0.12	[9]
HPLC-FL	GSH, hCys, Cys, AcCys	Blood	Deproteinization with EDTA and TCA; derivatization with IAB; mobile phase: methanol:15 mM Na ₂ HPO ₄ pH 2.7	0.05–25	0.0023	[10]
HPLC-MS	GSH, Cys, GSSH, hCys	Rat tissues	Deproteinization; gradient elution with TFA/acetonitrile	1.5–740	0.75	[11]
HPLC-MS/MS	hCys	Serum, plasma	Reduction with DTT; deproteinization; separation with C8; mobile phase: 50% methanol–0.1% HOAc	Up to 61.6	1.0	[26]
HPLC-ED	hCys	Blood	Reduction with TBP; separation with ESA MD C18; mobile phase: 0.15 M NaH ₂ PO ₄ , 1 mM SDS, 10% AcCN pH 2.9	Up to 500	0.14	[27]
HPLC-ED	hCys, GSH, Cys	Plasma	Separation with Intertsil ODS 2; mobile phase: 0.1 M MCA:methanol:DMF 93.25:5:1.75, 2.25 mM heptanesulfonic acid pH 2.8; detection at Au/Hg dual series electrodes	6.25–100	0.02	[12]
HPLC-ED	hCys	Plasma	Reduction with TCEP; deproteinization with HClO ₄ ; separation with ESA HR-80 C18; mobile phase: 0.15 M NaH ₂ PO ₄ , 1 mM SDS, 10% AcCN, pH 2.8	Up to 100	–	[1]
HPLC-PED	Met, Cys, HCys	–	Separation with Dionex AminoPac; waveform potential: 50 mV, 420 ms; 650 mV, 180 ms; –220 mV, 380 ms; 750 mV, 90 s	0.1–100 (Cys)	0.32	[20]
HPLC-ED	hCys, Cys, Met	Plasma	Reduction with NaBH ₄ ; deproteinization with HClO ₄ ; separation with Phenomenex Luna C18; mobile phase: 0.2 M PBS pH 3.0–5% methanol detection at Pt nanoparticles-oli(methyl violet) modified GCE	0.2–100	0.1	[22]

^a S/N = 3; ED, electrochemical detection; PED, pulsed electrochemical detection; Met, methionine; hCys, homocysteine; Cys, cysteine; Cys Gly, cysteine-glycine dipeptide; GSH, glutathion; GSSH, glutathion disulphide; TCEP, tris-(2-carboxyethyl)phosphine hydrochloride; CMTQ, 2-chloro-1-methylquinolinium tetrafluoroborate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; DTT, dithiotriol; mBBR, monobromobimane; TBP, tri-*n*-butylphosphine.

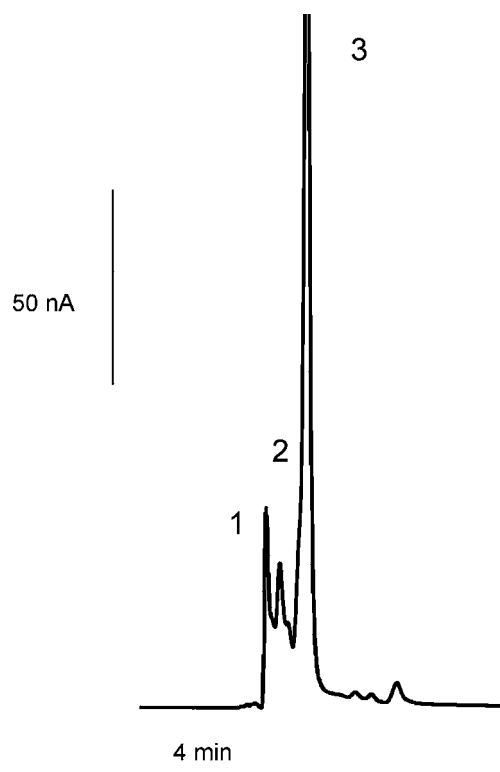


Fig. 7. Chromatogram obtained from a serum sample with amperometric detection at a Au_{coll} -Cyst-CPE: (1) cysteine; (2) homocysteine; (3) uric acid. Conditions as in Fig. 5.

mean concentration ($n=5$) for cysteine in the serum sample after spiking, of $5.3 \pm 0.3 \mu\text{M}$, which means a recovery of $96 \pm 5\%$. In the case of homocysteine, the concentration found in the serum sample after spiking was $8.4 \pm 0.4 \mu\text{M}$, that is a recovery of $104 \pm 5\%$.

4. Conclusions

The unique properties of gold nanoparticles permit the preparation of CP modified electrodes, which exhibit improved electrochemical oxidation responses of homocysteine with respect to conventional electrode materials. These enhanced electroanalytical performance in terms of sensitivity and stability allows the development of an HPLC method with amperometric detection at the Au_{coll} -Cyst-CPE for the separation and quantification of homocysteine and related thiols, that can be advantageously compared with other HPLC methods using electrochemical, UV, fluorescence and MS detection modes. Furthermore, the usefulness of this method has been demonstrated by applying it to the analysis of a serum sample with good results.

Congratulations

Dear Joe,

All we work on Analytical Electrochemistry must acknowledge your pioneer work and your innovative ideas on this

research field and last, but not least, your friendship and your capacity of cooperation with different groups all around the world. Happy birthday!



Acknowledgements

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References

- [1] G. Zhang, D. Liu, S. Shuang, M.M.F. Choi, *Sens. Actuators B* 114 (2006) 936.
- [2] H. Refsum, P.M. Ueland, O. Nygård, S.E. Vollset, *Annu. Rev. Med.* 49 (1998) 31.
- [3] D.A. Cramer, *Lab. Med.* 29 (1998) 410.
- [4] Y. Tan, L. Tang, X. Sun, N. Zhang, Q. Han, M. Xu, E. Baranov, X. Tan, X. Tan, B. Rashidi, Z. An, A.W. Perry, R.M. Hoffman, *Clin. Chem.* 46 (2000) 1686.
- [5] V. Ducros, K. Demuth, M.P. Sauvant, M. Quillard, E. Caussé, M. Candito, M.-H. Read, J. Drai, I. García, M.F. Gerhardt, *J. Chromatogr. B* 781 (2002) 207.
- [6] S.W. Myung, M. Kim, H.K. Min, E.A. Yoo, K.R. Kim, *J. Chromatogr. B* 727 (1999) 1.
- [7] K. Amarnath, V. Amarnath, K. Amarnath, H.L. Valentine, W.M. Valentine, *Talanta* 60 (2003) 1229.
- [8] E. Bald, E. Kaniowska, G. Chwatko, R. Glowacki, *Talanta* 20 (2000) 1233.
- [9] S.T. Chou, L.E. Ko, C.S. Yang, *Anal. Chim. Acta* 429 (2001) 331.
- [10] H. Wang, S.C. Liang, Z.M. Zhang, H.S. Zhang, *Anal. Chim. Acta* 512 (2004) 281.
- [11] X. Guan, B. Hoffman, C. Dwivedi, D.P. Matthees, *J. Pharm. Biomed. Anal.* 31 (2003) 251.
- [12] W.A. Kleinman, J.P. Richie Jr., *J. Chromatogr. B* 672 (1995) 73.
- [13] O. Nekrasova, N.S. Lawrence, R.G. Compton, *Talanta* 60 (2003) 1085.
- [14] A. Salimi, S. Pourbyram, *Talanta* 60 (2003) 205.
- [15] B. Svensmark, O. Hammerich, in: H. Lund, M.M. Baizer (Eds.), *Organic Electrochemistry. An Introduction and a Guide*, Marcel Dekker, NY, 1991, p. 660.
- [16] W.R. La Course, G.S. Owens, *Anal. Chim. Acta* 307 (1995) 301.
- [17] N.S. Lawrence, R.P. Deo, J. Wang, *Talanta* 63 (2004) 443.
- [18] G. Shi, J. Lu, F. Xu, W. Sun, L. Jin, K. Yamamoto, S. Tao, J. Jin, *Anal. Chim. Acta* 386 (1999) 21.
- [19] J. Wang, P.V.A. Pamidi, C. Parrado, D.S. Park, J. Pingarrón, *Electroanalysis* 9 (1997) 908.
- [20] T.R.I. Cataldi, D. Nardiello, *J. Chromatogr. A* 1066 (2005) 133.
- [21] K. Gong, Y. Dong, S. Xiong, Y. Chen, L. Mao, *Biosens. Bioelectron.* 10 (2004) 253.
- [22] H. Xu, W. Zhang, W. Zhu, D. Wang, J. Ye, K. Yamamoto, L. Jin, *Anal. Chim. Acta* 545 (2005) 182.

- [23] D. Hernández-Santos, M.B. González-García, A. Costa-García, *Electroanalysis* 14 (2002) 1225.
- [24] J. Manso, L. Agüí, P. Yáñez-Sedeño, J.M. Pingarrón, *Anal. Lett.* 37 (2004) 901.
- [25] L. Agüí, J. Manso, P. Yáñez-Sedeño, J.M. Pingarrón, *Talanta* 64 (2004) 1041.
- [26] J. Kuhn, C. Götting, K. Kleesiek, *Clin. Biochem.* 39 (2006) 164.
- [27] R. Accinni, S. Bartesaghi, G. De Leo, C.F. Cursano, G. Achilli, A. Loaldi, C. Cellerino, O. Parodi, *J. Chromatogr. A* 896 (2000) 183.

Voltammetric determination of aminobiphenyls at a boron-doped nanocrystalline diamond film electrode

Jiri Barek^{*}, Kateřina Jandová, Karolina Pecková, Jiří Zima

Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry, 128 43 Prague 2, Czech Republic

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Dedicated to Professor Joseph Wang on the occasion of his 60th birthday.

Abstract

Voltammetric behavior of 2-aminobiphenyl, 3-aminobiphenyl, and 4-aminobiphenyl at a boron-doped nanocrystalline diamond film electrode was investigated using cyclic voltammetry and differential pulse voltammetry. Optimum conditions have been found for the determination of those genotoxic substances by differential pulse voltammetry at the above given electrode in the concentration range of 2×10^{-7} to 1×10^{-5} mol/L.

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Keywords: Boron-doped nanocrystalline diamond film electrode; Differential pulse voltammetry; Cyclic Voltammetry; 2-Aminobiphenyl; 3-Aminobiphenyl; 4-Aminobiphenyl

1. Introduction

Aminobiphenyls are among proven (4-aminobiphenyl) or suspected chemical carcinogens [1]. They can be found in combustion products of fossil fuels and in cigarette smoke. They are metabolized forming unstable nitrenium ion capable to react with nucleophilic centers of DNA [2] thus causing human bladder cancer. Moreover, they are products of metabolic transformation of nitrobiphenyls and thus they can serve as biomarkers of human exposure to carcinogenic nitrobiphenyls [3]. Therefore, there is an ever-increasing demand for the determination of trace amounts of these dangerous substances. Among most frequently used methods are HPLC with spectrophotometric or MS [4] detection. GC–MS [5] and spectrofluorimetry [6] are other extremely sensitive methods for aminobiphenyls. However, these methods are relatively expensive, instrumentally demanding and time consuming. Therefore, we pay attention to voltammetric determination of various genotoxic substances, because electroanalytical methods are

extremely sensitive, sufficiently selective, and inexpensive and thus suitable for large scale monitoring of various environmental pollutants [7]. Aminobiphenyls are easily electrochemically oxidisable and thus they can be determined by differential pulse voltammetry at solid or carbon paste electrodes [8]. However, nearly all sp^2 carbon electrodes are susceptible to strong adsorption of polar species mainly due to the presence of polar functionalities on the surface [9]. This problem can be overcome by using a new electrode material which is rapidly gaining popularity—boron-doped diamond film [10–15]. Among advantages of boron-doped nanocrystalline diamond film electrode (BDNDFE) are a low and stable background current over a wide potential range, microstructural and morphological stability at extreme cathodic and anodic potentials, hydrophobic effect of hydrogen terminated diamond surface, very low adsorption of polar molecules, and chemical inertness. We have recently successfully used this electrode material for voltammetric determination of both oxidisable and reducible carcinogens [15,16]. The aim of this paper is to show that BDNDFE is a suitable sensor for the determination of trace amounts of aminobiphenyls which can passivate glassy carbon electrodes thus complicating their use for voltammetric determination of those genotoxic compounds.

^{*} Corresponding author. Tel.: +420 221951224; fax: +420 224913538.
E-mail address: Barek@natur.cuni.cz (J. Barek).

2. Experimental

2.1. Reagents

Stock solutions of 2-aminobiphenyl (2-AB), 3-aminobiphenyl (3-AB), and 4-amino-biphenyl (4-AB) ($c = 1 \times 10^{-4}$ mol/L) were prepared by dissolving precisely weighed amount of the substances supplied by Sigma–Aldrich in 250 mL of deionized water using sonication. Solutions of lower concentrations were prepared by dilution of stock solution with deionized water. All solutions were kept in darkness. Sodium hydroxide, glacial acetic acid, boric acid, and potassium hexacyanoferrate (all p.a. purity) were obtained from Lachema Brno (Czech Republic). Propan-2-ol (for spectroscopy), methanol, and acetonitrile (for chromatography) were supplied by Merck, Darmstadt. Deionized water from Millipore Q-plus System (USA) was used for all experiments.

2.2. Apparatus

All measurements were carried out using a computer driven Eco-Tribo Polarograph with PolarPro software, version 4.0 (both Polaro-Sensors, Czech Republic) with working nanocrystalline boron-doped diamond thin-film electrode constructed in our laboratory of an active surface of 12.6 mm^2 in a disk arrangement described in ref. [16]. Chemical vapour deposition (CVD) growing conditions, boron-doping level, film resistivity, etc., are described in ref. [23]. The nanocrystalline film was deposited by microwave-assisted chemical vapour deposition using a $\text{CH}_4/\text{H}_2/\text{Ar}$ source gas mixture (1%/5%/95%), the boron-doping level being around $5 \times 10^{20} \text{ B/cm}^3$ and the apparent resistivity was $0.1 \Omega \text{ cm}$. The good performance of the electrode was regularly verified by measuring cyclic voltammograms of 1×10^{-4} mol/L potassium hexacyanoferrate. With BDNDFE freshly cleaned with propan-2-ol, the difference between anodic and cathodic peak potential was from 105 to 265 mV and the ratio of $I_p \text{ red}/I_p \text{ ox}$ was from 1.00 to 1.08 at scan rates from 20 to 500 mV/s. When these values have changed for more than 10% from the original one, the electrode was carefully cleaned by immersion into propan-2-ol under stirring. The reference was argentchloride (1 M KCl) electrode (type RAE 113), to which all the potentials were referred, and platinum wire auxiliary electrode PPE (both from Monokrystaly, Turnov, Czech Republic) were used. In differential pulse voltammetry the pulse amplitude +50 mV and the width of pulse 100 ms were applied. Unless stated otherwise, a polarization rate 20 mV/s was used. The pH measurements were carried out by Conductivity & pH meter Jenway 4330 with combined glass electrode (Ag/AgCl/KCl, 3 mol/L) (type 924005) (all Jenway, United Kingdom).

2.3. Procedures

The general procedure to obtain cyclic or differential pulse voltammograms was as follows: the required amount of the aqueous stock solution of the test substance was placed in a 10 mL volumetric flask, the solution was diluted to the mark

with a Britton–Robinson buffer of the required pH, transferred to the voltammetric vessel, bubbled with nitrogen for 15 s and the voltammogram was recorded. Between individual measurements, the measured solution was always bubbled with nitrogen for 15 s. Between measurements of different solutions, the electrode was always washed with deionized water, immersed into methanol which was bubbled with nitrogen for 1 min and again washed with deionized water. When the electrode did not perform well, it was activated by immersion into propan-2-ol for 20 min without stirring. The calibration curves were measured three times and all experiments were carried out under laboratory temperature. The statistical parameters of calibration dependences (i.e. slope, intercept, limit of determination) were calculated according to Oppenheimer [17], Schwartz [18], and Ebel [19] using statistic software ADSTAT version 2.0 (Trilobyte, Czech Republic). This software used confidence bands ($\alpha = 0.05$) for calculation of the limit of determination (L_Q). It corresponded to the lowest signal for which relative standard deviation (R.S.D.) is equal to 0.1 [20].

3. Results and discussion

First, the influence of pH on DPV curves of 1×10^{-5} mol/L 2-AB at BDNDFE was investigated in Britton–Robinson buffer, pH 2–12 (see Fig. 1). The substance gives four peaks in the region of pH 2–5 (first two peaks are not completely separated), two peaks at pH 6 (first and third from the set observed at lower pH) and one peak at pH 7–12 (third peak from those observed in acidic medium). The dependence of peak potentials on pH is depicted in Fig. 2. The observed shift of peak potentials towards positive values with decreasing pH can be explained by protonation of nitrogen atom causing the decrease in electron density which results in more difficult oxidation. For analytical purposes, the most suitable is the third peak which is best developed at pH 7 (see Fig. 1). Voltammograms were recorded three times and solutions were always bubbled for 15 s between recordings to obtain best repeatable data. The height of the peak was measured from the tangent of voltammetric curve before and after the peak. Linear concentration dependencies were obtained under these conditions within the concentration

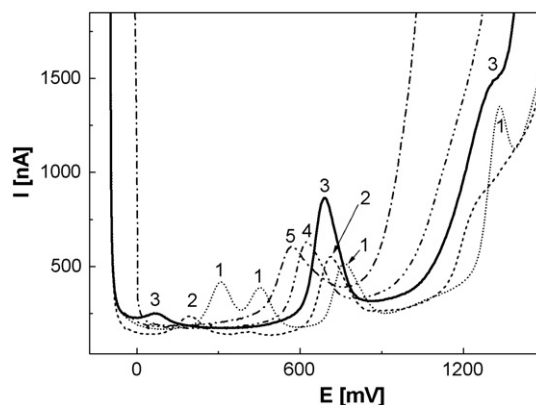


Fig. 1. Selected DP voltammograms of 2-AB ($c = 1 \times 10^{-5}$ mol/L) at BDNDFE in Britton–Robinson buffer pH 3.0 (1), 5.0 (2), 7.0 (3), 9.0 (4), and 11.0 (5).

Table 1

Parameters of calibration straight lines for determination of aminobiphenyls by DPV at BDNDFE in BR buffer

Substance	pH	c (mol/L)	Slope (nA mol ⁻¹ L)	Intercept (nA)	Correlation coefficient	L_Q (mol/L)
2-AB	7.0	$(2-10) \times 10^{-6}$	4.19×10^7	54.54	0.9988	–
		$(1-10) \times 10^{-7}$	6.33×10^7	0.92	0.9988	1.2×10^{-7}
3-AB	8.0	$(2-8) \times 10^{-6}$	2.81×10^7	18.33	0.9998	–
		$(2-10) \times 10^{-7}$	3.36×10^7	4.98	0.9988	1.3×10^{-7}
4-AB	9.0	$(2-10) \times 10^{-6}$	3.24×10^7	26.01	0.9968	–
		$(1-10) \times 10^{-7}$	4.33×10^7	3.92	0.9976	2.5×10^{-7}

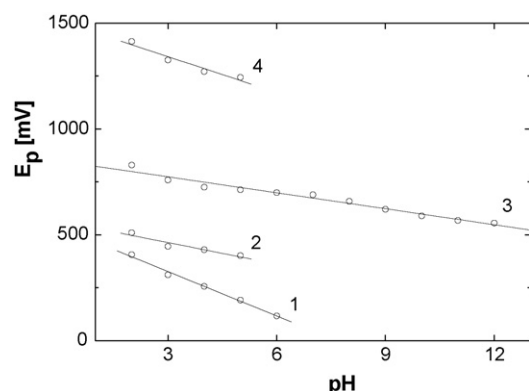


Fig. 2. Dependence of peak potential E_p of 2-AB ($c = 1 \times 10^{-5}$ mol/L) on pH. Measured by DPV at BDNDFE in BR buffer, 1: first peak, 2: second peak, 3: third peak, 4: fourth peak.

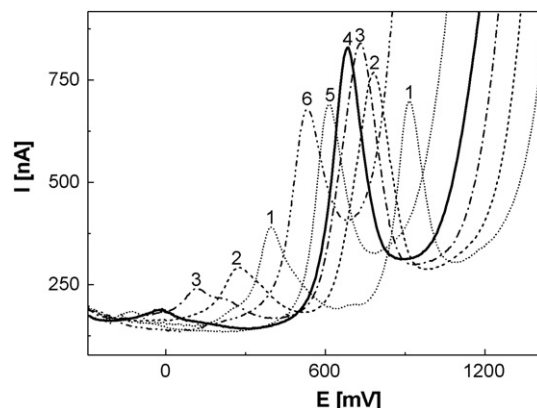


Fig. 4. Selected DP voltammograms of 3-AB ($c = 1 \times 10^{-5}$ mol/L) at BDNDFE, in Britton–Robinson buffer pH 2.0 (1), 4.0 (2), 6.0 (3), 8.0 (4), 10.0 (5), and 12.0 (6).

range of $(1-100) \times 10^{-7}$ mol/L (corresponding voltammograms are depicted in Fig. 3a for the sake of illustration). Parameters of those dependencies are summarized in Table 1. The calibration dependency is not linear in the whole concentration range which results in larger intercept of dependencies for higher concentrations. Voltammograms at concentrations lower than 1×10^{-7} mol/L could not be evaluated. Repeatability of the determination at 1×10^{-7} mol/L concentration level is characterized by relative standard deviation 10%, while at concentration 1×10^{-5} mol/L the relative standard deviation was 1.6%.

3-AB gives only two peaks, the first one decreasing with increasing pH (see Fig. 4). Again, the peak potentials shift

towards positive values with decreasing pH. The best-developed second peak was obtained at pH 8 (see Fig. 4). In this medium, the linear concentration dependence was obtained within concentration range $(2-80) \times 10^{-7}$ mol/L, i.e. slightly narrower than in the case of 2-AB. This is probably connected with more pronounced passivation of electrode surface resulting in lower signal and higher limit of detection. In this case we could observe the decrease of peak heights after repeated measurements. This is probably connected with formation of a polyamine film at the surface of BDNDFE. This fact is supported by cyclic voltammograms (see Fig. 5). Peak p^a corresponds to irreversible oxidation of 3-AB while peaks p^{c1} , p^{c2} , p^{a2} , and p^{a1} , which form two quasireversible pairs, at lower potentials obviously correspond

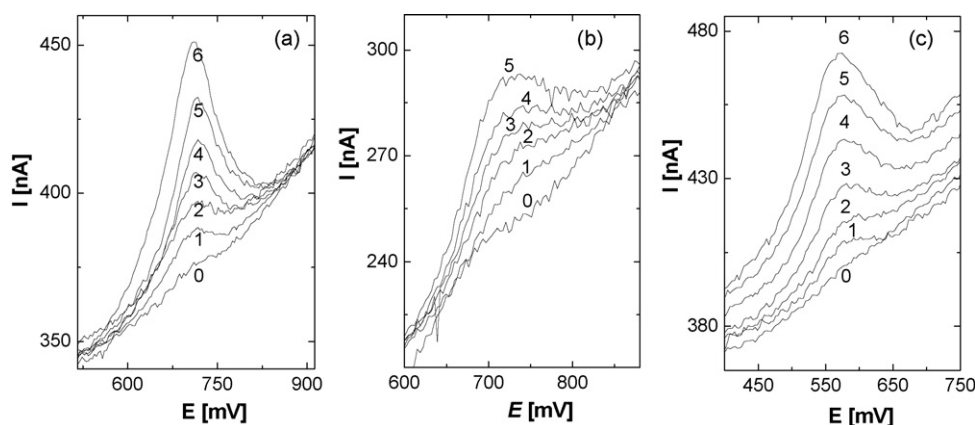


Fig. 3. Voltammograms of 2-AB (a), 3-AB (b), and 4-AB (c) measured by DPV at BDNDFE in BR buffer pH 7 (a), pH 8 (b), and pH 9 (c). $c(\text{AB}) = 0$ mol/L (0), 1×10^{-7} mol/L (1), 2×10^{-7} mol/L (2), 4×10^{-7} mol/L (3), 6×10^{-7} mol/L (4), 8×10^{-7} mol/L (5), and 1×10^{-6} mol/L (6).

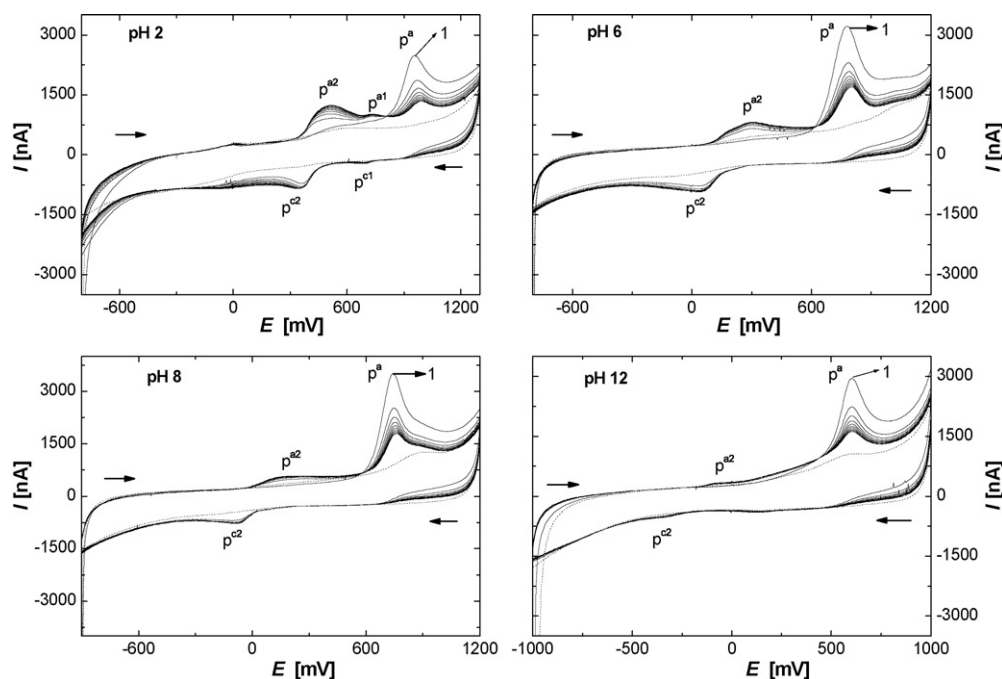


Fig. 5. Cyclic voltammograms of 3-AB ($c = 1 \times 10^{-5}$ mol/L) measured at BDNDFE at scan rate 100 mV/s in BR buffer of different pH. Dotted line corresponds to the base electrolyte. 1: first scan.

to reaction products. Pair of peaks p^{c1} and p^{a1} is observable only at pH 2. All peaks shift to lower potentials with increasing pH. Peaks p^{c2} and p^{a2} are decreasing with increasing pH probably due to higher solubility of polyamines formed at the electrode surface in an alkaline media [21].

We have found that cleaning the electrode surface with methanol practically eliminates problems with passivation of the electrode in contrary with electrochemical treatment or washing with water (see Fig. 6). The inefficiency of electrochemical cleaning is rather surprising in connection with observed easy electrochemical removal of a polyaniline film from the surface of BDNDFE [21]. However, submicromolar concentrations of 3-AB still give well developed voltammetric peaks and linear calibration curve (see Fig. 3b for corresponding voltammo-

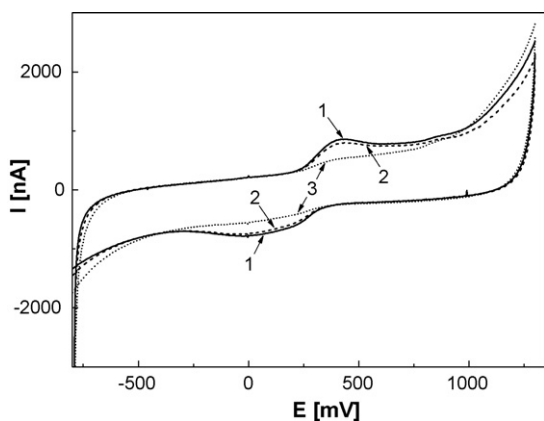


Fig. 6. Cyclic voltammograms of polyamine films formed in 1×10^{-5} mol/L 3-AB in BR buffer pH 6 documenting effectiveness of various ways of electrode cleaning; 1: washing with water; 2: electrochemical cleaning at +2.0 V for 5 min; 3: immersion in methanol and bubbling with nitrogen for 15 s.

grams). Parameters of obtained calibration straight lines are again summarized in Table 1. Relative standard deviation for determination of 2×10^{-7} mol/L 3-AB was 5.2%, whereas for 8×10^{-6} mol/L (the upper point of linear dynamic range) was equal to 2.8%.

DP voltammetric behavior of 4-AB is somewhat more complicated. The substance gives four peaks at pH 4, three peaks at pH 3–4, one badly developed peak at pH 5–7 and only one, relatively well developed peak at pH 8–12 (see Fig. 7). Dependence of peak potentials on pH is depicted in Fig. 8. First change of the slope for the third peak obviously corresponds to pK_a of 4-AB (tabulated value is 4.22). The second change is not quite clear at the moment. Probably it is connected with acid–base behavior of oxidation products.

From the analytical point of view, the best-developed peak was obtained at pH 9 (see Fig. 7) where well developed peaks

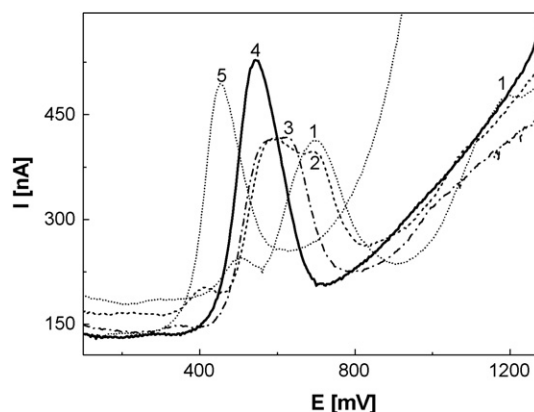


Fig. 7. Selected DP voltammograms of 4-AB ($c = 1 \times 10^{-5}$ mol/L) at BDNDFE, in Britton–Robinson buffer pH 3.0 (1), 5.0 (2), 7.0 (3), 9.0 (4), and 11.0 (5).

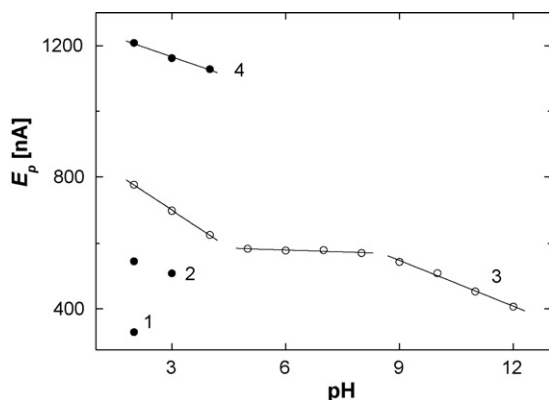


Fig. 8. Dependence of peak potential E_p of 4-AB ($c = 1 \times 10^{-5}$ mol/L) on pH. Measured by DPV at BDNDFE in BR buffer, 1: first peak, 2: second peak, 3: third peak, 4: fourth peak.

and linear concentration dependence were obtained in submicromolar concentration range (for corresponding voltammograms see Fig. 3c). Parameters of calibration straight lines are given in Table 1. Relative standard deviation for the determination of 2×10^{-7} mol/L 3-AB was 12.7%, whereas for 1×10^{-5} mol/L it was equal to 2.9%.

Somewhat different behavior of the three tested positions isomers is probably connected with different structure of corresponding electrochemically produced polyamines analogously to previously studied oxidation of aniline at BDNDFE [21] and *p*-aminobiphenyl at traditional electrode materials [22]. In the case of 4-AB, the conventional C–N head-to-tail coupling is presumed at a platinum electrode [22] leading to linear polymers in which biphenyl groups are incorporated in the main macromolecular chain. The structure of electropolymerized 2-AB and 3-AB is obviously somewhat different. Moreover, C–C phenyl–phenyl coupling can occur in the case of 2-AB and 3-AB, similarly to diphenylamine electropolymerization [22].

We have preliminary tested the practical application of the newly developed method on model samples of investigated aminobiphenyls in drinking water, the obtained LOD's being comparable with those summarized in Table 1. However, it follows from our preliminary experiments that the practical application on more complex model samples of river or surface water will require a preliminary separation using solid phase extraction. Probably the same will hold for practical application on blood and urine samples for biological monitoring of exposition to nitrated biphenyls. This topic is now under investigation in our laboratory.

4. Conclusions

The observed decrease of sensitivity with increasing concentration of aminobiphenyls is probably connected with the formation of polyamine films at the surface of working electrode. This is further confirmed by broader and less symmetrical peaks of aminobiphenyls as compared with hexacyanoferrate as a model substance. However, this passivation does not prevent analytical utilization of BDNDFE for voltammetric determi-

nation of those substances. Simple immersion of BDNDFE in methanol eliminates most problems with electrode passivation in the case of investigated substances. In the case of more severe problems with passivation, the application of propan-2-ol is even more efficient. Observed limits of determination of tested aminobiphenyls by DPV at BDNDFE are somewhat lower than for DPV at a carbon paste electrode [8]. It can be expected that the use of mixed aqueous-methanolic solutions, in which the formed polyamines could be dissolved, would further diminish problems with passivation and the preliminary results of our ongoing research confirm this assumption.

Congratulation

Dear Joe, on behalf of all your friends in the Czech Republic, a cradle of polarography, we wish you many happy returns, good health, many new fascinating ideas to explore, a lot of success in developing new sensitive and selective analytical procedures, many new, exciting materials to study, new books on electroanalytical chemistry, many enthusiastic and creative coworkers and students and success and happiness both in scientific career and in private life. We take this opportunity to thank you for a fruitful cooperation with our laboratory and for accepting our PhD students and postdocs in your excellent laboratory thus helping them to start their successful scientific career.

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References

- [1] Anon., International Agency for Research on Cancer (IARC), IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, vol. 1, WHO, Lyon, 1972.

- [2] Z. Feng, W. Hu, W.N. Rom, F.A. Beland, *Carcinogenesis* 23 (2002) 1721.
- [3] J.C. Moreira, J. Barek, *Quim. Nova* 18 (1995) 362.
- [4] R.J. Turesky, J.P. Freeman, R.D. Holland, D.M. Nestorick, D.W. Miller, D.L. Ratnasinghe, F.F. Kadlubar, *Res. Toxicol.* 16 (2003) 1162.
- [5] R. Stabbert, K.H. Schäfer, C. Biefel, K. Rustemeier, *Rapid Commun. Mass Spectrom.* 17 (2003) 2125.
- [6] C.L. Holder, J.R. King, M.C. Bowman, *J. Toxicol. Environ. Health* 2 (1976) 111.
- [7] J. Wang, *Analytical Electrochemistry*, 2nd ed., Wiley–VCH, New York, 2000.
- [8] J. Barek, J. Cvačka, A. Muck, V. Quaiserová, J. Zima, *Electroanalysis* 13 (2001) 799.
- [9] R.L. McCreery, in: A. Wieckowski (Ed.), *Interfacial Electrochemistry*, Dekker, New York, 1999, p. 627.
- [10] A. Fujishima, Y. Einaga, T.N. Rao, D.A. Tryk, *Diamond Electrochemistry*, Elsevier, Amsterdam, 2005.
- [11] R.G. Compton, J.S. Foord, F. Marken, *Electroanalysis* 15 (2003) 1349.
- [12] Y.V. Pleskov, *Russ. J. Electrochem.* 38 (2002) 1275.
- [13] Y.V. Pleskov, *Prot. Met.* 42 (2006) 115.
- [14] Y.V. Chailapakul, W. Siangproh, D.A. Tryk, *Sens. Lett.* 4 (2006) 99.
- [15] J. Barek, J. Fischer, T. Navrátil, K. Pecková, B. Yosypchuk, J. Zima, *Electroanalysis*, in press.
- [16] K. Cizek, J. Barek, J. Fischer, K. Peckova, J. Zima, *Electroanalysis* 19 (2007) 1295.
- [17] L. Oppenheimer, T.P. Cappizi, R.M. Weppelmann, H. Metha, *Anal. Chem.* 55 (1983) 638.
- [18] L.M. Schwartz, *Anal. Chem.* 55 (1983) 1424.
- [19] S. Ebel, U. Kamm, *Fresenius J. Anal. Chem.* 318 (1984) 293.
- [20] M. Meloun, J. Militký, M. Forina, *Chemometrics for Analytical Chemistry, PC-aided Regression and Related Methods*, vol. 2, Ellis Horwood, Chichester, 1992, pp. 1–175.
- [21] M. Mitadera, N. Spataru, A. Fujishima, *J. Appl. Electrochem.* 34 (2004) 249.
- [22] J. Guay, L.H. Dao, *J. Electroanal. Chem.* 274 (1989) 135.
- [23] Y. Show, M.A. Witek, P. Sonthalia, G.M. Swain, *Chem. Mater.* 15 (2003) 879.

Review

Carbon nanotube and diamond as electrochemical detectors in microchip and conventional capillary electrophoresis

Gang Chen*

Department of Analytical Chemistry, School of Pharmacy, Fudan University, Shanghai 200032, China

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Abstract

As two important polymorphs of carbon, carbon nanotube (CNT) and diamond have been widely employed as electrode materials for electrochemical sensing. This review focuses on recent advances and the key strategies in the fabrication and application of electrochemical detectors in microchip and conventional capillary electrophoresis (CE) using CNT and boron-doped diamond. The subjects covered include CNT-based electrochemical detectors in microchip CE, CNT-based electrochemical detectors in conventional CE, boron-doped diamond electrochemical detectors in microchip CE, and boron-doped diamond electrochemical detectors in conventional CE. The attractive properties of CNT and boron-doped diamond make them very promising materials for the electrochemical detection in microchip and conventional CE systems and other microfluidic analysis systems.

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Keywords: Carbon nanotube; Boron-doped diamond; Microchip; Capillary electrophoresis; Amperometry; Electrochemical detector

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1. Introduction

Since capillary electrophoresis (CE) in its modern form was first described by Jorgenson and Luckas in 1981 [1,2], the application of CE has become increasingly widespread because of its minimal sample volume requirement, short analysis time, and

high separation efficiency. Microfluidic analytical systems fabricated on silica, glass, and polymer microchips have undergone an explosive growth during the past decade. Microchip CE have received much attention because of its high degree of integration, portability, minimal solvent/reagent consumption, high performance, and speed. Both microchip and conventional CE hold considerable promise for biomedical and pharmaceutical analysis, clinical diagnostics, environmental monitoring, and forensic investigations [3–6].

* Tel.: +86 21 6466 1130; fax: +86 21 6564 1740.

E-mail address: gangchen@fudan.edu.cn.

Optical, electrochemical and mass spectrometric detection modes have become routine for microchip and conventional CE. Electrochemical detection (ECD) offers great promise for such microsystems, with features that include remarkable sensitivity, inherent miniaturization of both the detector and control instrumentation, independence of sample turbidity or optical path length, low cost, minimal power demands, and high compatibility [7–9]. The performance of microchip and conventional CE-ECD is strongly influenced by the detection-electrode material. The detection electrode should provide not only favorable signal-to-background characteristics but also a reproducible response. A range of materials, including platinum, gold, and various forms of carbon, have thus been found useful for microfluidic electrochemical detection [10–13]. CNT and diamond are two important polymorphs of carbon and have been widely employed as electrode materials for electrochemical sensing. Since Iijima reported the existence of CNT in 1991 [14], they have attracted much attention because of their high electrical conductivity, mechanical strength, and chemical stability [15,16]. The unique properties of CNT make it extremely attractive for electrochemical sensors and biosensors [17–19]. Recent investigations demonstrated that CNT shows strong electrocatalytic activity and minimization of surface fouling when it was employed to improve the electrochemical response of some important bioactive substances [20–23]. Although diamond is an electrical insulator, it becomes an electric conductor when it is doped by some elements such as boron, nitrogen, etc. Boron-doped diamond (BDD) electrodes own attractive properties such as a wide potential window, low and stable background currents, negligible adsorption of organic compounds, and low noise. They have been widely used for detecting nucleic acids [24], cysteine [25], azide anion [26], NADH [27], chlorophenols [28], cytochrome C [29], etc. Both CNT and boron-doped diamond have been employed to fabricate the electrochemical detectors for microchip and conventional CE systems because of their unique attributes.

This review focuses on recent advances in the application of CNT and boron-doped diamond to the electrochemical detection of microchip and conventional CE. These fields of study have grown considerably in the past decade. Reports ranging from the fabrication to the application of CNT and boron-doped diamond detectors have been published. The following sections will cover CNT-based electrochemical detectors in microchip CE, CNT-based electrochemical detectors in conventional CE, boron-doped diamond electrochemical detectors in microchip CE, and boron-doped diamond electrochemical detectors in conventional CE.

2. CNT-based electrochemical detectors in CE

A variety of CNT-based electrodes have been fabricated for sensing different electroactive compounds, including CNT modified screen-printed carbon electrode [30], CNT/nano-Fe₃O₄ microelectrode [31], CNT/Teflon composite electrode [32], CNT screen-printed electrode [33], CNT/epoxy composite electrode [34], CNT/copper composite electrode [35], etc. The commonly used approach for fabricating CNT electrodes is sur-

face modification. To load CNT on the surface of electrodes, CNTs were usually dispersed in solvents [36,37] or polymer solutions (such as Nafion [38] and chitosan [39] solutions) that were cast on the electrodes because CNT was insoluble in most solvents. Recently, methods based on electrochemical deposition [40] and electrochemical polymerization [41,42] have been employed for loading CNT on electrodes. CNT-based electrodes have been demonstrated to reduce the overpotential significantly [17,40]. The abilities of CNT to promote the electron-transfer reactions and to minimize the surface fouling suggest great promise for amperometric detection of microchip and conventional CE [17].

2.1. CNT-based electrochemical detectors in microchip CE

Wang et al. have modified CNT on thick-film carbon electrodes by casting 0.5%, w/w Nafion (in ethanol) containing 2 mg/mL CNT that was allowed to dry at room temperature [30]. The CNT-modified electrode has been coupled with microchip CE system as an amperometric detector. It allowed CE amperometric detection at significantly lower operating potentials and yielded substantially enhanced S/N characteristics. The electrocatalytic detection was coupled to resistance to surface fouling and hence enhanced reproducibility. Such advantages were demonstrated by the separation and detection of hydrazine, phenol, purine, and amino acid compounds. Substantial minimization of surface fouling effects has been demonstrated in connection with the monitoring of phenol and tyrosine. CNTs from different sources were evaluated by the detection of dopamine and catechol. The broad and significant catalytic activity exhibited by CNT-based CE detectors indicates great promise for a wide range of bioanalytical and environmental applications.

Recently, a novel single wall carbon nanotube (SWCNT) modified gold detector for microchip CE has been developed by Pumera et al. for the detection of *p*-aminophenol, *o*-aminophenol, dopamine and catechol [43]. The gold working electrode was modified by casting SWCNT dispersed in *N,N*-dimethyl formamide (DMF) and drying at room temperature. SWCNT modified gold electrode displayed greatly improved sensitivity and separation resolution compared to bare gold electrode, indicating the electrocatalytic activity of SWCNT.

Vlckova et al. have modified multi-wall carbon nanotube (MWCNT) on gold electrode in the same way for the simultaneous determination of catecholamines and their *O*-methoxylated metabolites after separation by microchip electrophoresis [44]. The CNT-modified working electrode was employed for the sensitive amperometric detection of the analytes with submicromolar detection limits. The suitability of the CNT modified gold electrode for measuring low levels of catecholamines and their *O*-methoxylated metabolites has been demonstrated by analyzing mouse brain homogenate by microchip CE and human urine by conventional CE.

In 2004, Wang et al. have fabricated MWCNT/copper composite electrodes based on co-mixing CNT and Cu powders within mineral oil [35]. The new composite electrode was employed for the improved amperometric detection of car-

bohydrates (glucitol, glucose, gluconic acid, and glucuronic acid) following their microchip CE separations. The CNT/Cu composite electrode detector displayed enhanced sensitivity compared to detectors based on copper or CNT alone. The marked catalytic performance of the CNT/Cu composite material permitted low detection potential (+0.5 V *versus* Ag/AgCl) amperometric detection, and was coupled to the renewability, bulk modification and versatility advantages of composite electrodes. The CNT/Cu composite surface also led to a greater resistance to surface fouling compared to that observed at the copper electrode. The CNT/Cu composite electrode was also demonstrated to be useful for the detection of amino acids (arginine, histidine, and glycine) as indicated from preliminary results. While this work focused on the enhanced CE microchip detection of carbohydrates and amino acids, it is expected that the CNT/metal-composite electrode route should benefit the detection of other important groups of analytes.

More recently, we reported the development and application of a novel CNT/PMMA composite electrode as a sensitive amperometric detector of microchip CE [45]. The composite electrode was fabricated by the *in situ* polymerization of a mixture of MWCNT and prepolymerized methyl methacrylate in the microchannel of a piece of fused silica capillary under heat. The performance of this unique system was demonstrated by the separation and detection of phenolic pollutants and purines. The new CNT-based CE detector offered significantly reduced detection potentials and substantially enhanced S/N characteristics. In addition, the surface fouling was minimized so that the stability of the detector was improved. Long-term stability and reproducibility with relative standard deviation (R.S.D.) of less than 5% for the peak current ($n=20$) were also demonstrated. The simplicity and significant performance exhibited by the CNT/PMMA composite electrode indicated great promise for conventional CE, flowing-injection analysis, and other microfluidic analysis systems.

Shown in Fig. 1 are representative electropherograms for a mixture containing 2,6-dimethylphenol (a), phenol (b), 2-naphthol (c), 4-chlorophenol (d), pentachlorophenol (e), 2,4-chlorophenol (f), 3-nitrophenol (g), and 4-nitrophenol (h) recorded with graphite/PMMA (A) and CNT/PMMA (B) composite electrode detectors. The eight phenols can be separated resulting in well-defined and resolved peaks with the CNT/PMMA composite electrode within 200 s. Broader peaks (and hence inferior resolution) are observed at the graphite/PMMA detector. The sharp and well-resolved responses at the CNT/PMMA detector result in smaller values of the half-peak widths for 2,6-dimethylphenol, phenol, 2-naphthol, 4-chlorophenol, pentachlorophenol, 2,4-chlorophenol, 3-nitrophenol, and 4-nitrophenol at the CNT/PMMA electrode when compared to graphite/PMMA electrode (3.9 *versus* 5.1, 3.9 *versus* 4.5, 3.9 *versus* 4.6, 3.8 *versus* 4.8, 4.4 *versus* 5.5, 4.2 *versus* 5.1, 4.9 *versus* 7.3, and 5.8 *versus* 9.1 s, respectively).

The present CNT/PMMA composite electrode shows low-noise characteristics and stable baseline, which is not always the case for CNT coated [30,43] and CNT paste electrodes [35,46] employed as detectors in conventional and microchip

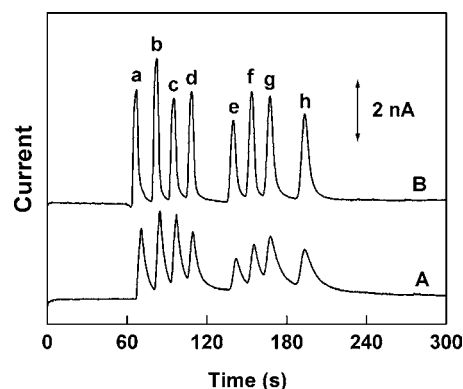


Fig. 1. Electropherograms for a mixture containing 50 μ M 2,6-dimethylphenol (a), 50 μ M phenol (b), 50 μ M 2-naphthol (c), 50 μ M 4-chlorophenol (d), 100 μ M pentachlorophenol (e), 100 μ M 2,4-chlorophenol (f), 100 μ M 3-nitrophenol (g), and 100 μ M 4-nitrophenol (h) at the graphite/PMMA (A) and the CNT/PMMA (B) composite microdisc electrodes. The separation channel in the glass CE microchip, 50 μ m wide $\times$ 20 μ m deep $\times$ 74 mm long; separation and injection voltage, +2500 V; injection time, 3 s; running buffer, 40 mM borate buffer (pH 9.8); detection potential, +0.8 V (*vs.* Ag/AgCl wire) (reprinted with permission [45]).

CE systems. It was demonstrated recently that CNT films on any electrodes surfaces provided higher noise level than bare electrodes in microfluidic systems [47]. In contrary, the PMMA/CNT electrode provided very nice S/N characteristics. The detection limits of 0.011 μ M 2,6-dimethylphenol, 0.009 μ M phenol, 0.012 μ M 4-chlorophenol, and 0.024 μ M 2,4-dichlorophenol were estimated based on a S/N ratio of 3. This CNT/PMMA electrode has a significant advantage over other CNT electrodes mentioned above because it is rigid and stable and is not prone to wearing over time. Its low noise level may be attributed to the stable and rigid conductive network of the CNT/PMMA composite. The enhanced current response of CNT/PMMA composite electrode can be ascribed to not only the electrocatalytic activity of CNT, but also the higher surface area of the CNT composite.

The exact reason for the sharper and well-resolved peaks observed at the wall-jet CNT-based detection electrodes in microchip CE systems was not previously fully understood [30–45]. Recently, Pumera et al. have demonstrated that a faster electron transfer on the surfaces of the electrocatalytic CNT electrodes coupled with a large volume of μ CE wall-jet electrochemical detectors offered explanation to this phenomenon [48].

2.2. CNT-based electrochemical detectors in conventional CE

Only few studies have addressed the development and the application of CNT-based electrodes for the electrochemical detection of conventional CE. Chicharro et al. have developed CNT paste electrodes (CNTPE) as the detectors for conventional CE [46]. CNTPE were fabricated by packing a paste mixture containing CNT powder and mineral oil firmly into the cavity of a Teflon tube. The classical carbon (graphite) paste electrode (CPE) was also prepared in a similar way by mixing graphite

powder with mineral oil. Compared to the classical CPE, CNTPE improved the detection limits of dopamine, epinephrine, norepinephrine, ascorbic acid, and 3,4-dihydroxyphenyl acetic acid (1.8 *versus* 3.3, 2.1 *versus* 4.4, 2.8 *versus* 5.3, 3.3 *versus* 17.7, and 13.3 *versus* 106 μM , respectively) at the detection potential of 400 mV. Hydrodynamic voltammograms corresponding to the electrophoretic separation of the five analytes were obtained at CNTPE and CPE. As expected, the oxidation currents of the analytes at CNTPE are shifted towards lower potentials with an enhancement in the peak currents indicating the electrocatalytic performance of CNT. The reproducibility of the signals obtained at CNTPE was evaluated from a series of 10 consecutive analyses. In all cases, the R.S.D.s were lower than 5%, indicating the high reproducibility and stability of CNTPE signals and their suitability for electrochemical detection in CE.

Yao et al. have employed the CNT/PMMA composite electrode mentioned above as a sensitive amperometric detector of conventional CE [49]. The performance of this unique system has been demonstrated by separating and detecting honokiol and magnolol in traditional Chinese medicine, *Cortex Magnoliae Officinalis*. Factors influencing their separation and detection processes were examined and optimized. Honokiol and magnolol were well separated within 7 min in a 40 cm long capillary at a separation voltage of 15 kV using a 50 mM borate buffer (pH 9.2). The new CNT-based CE detector allowed a low detection potential of +0.8 V (*versus* Ag/AgCl wire), yielding substantially enhanced S/N ratio compared to graphite/PMMA composite electrode. It offered long-term stability and reproducibility with R.S.D. of less than 5% for the peak current ($n=9$).

Chen et al. have fabricated CNT/epoxy composite electrodes as the detector of a specially designed miniaturized CE-amperometric detection system for the determination of several bioactive thiols [34]. To prepare such rigid CNT-based electrode, MWCNT-epoxy composite was packed into a piece of fused silica capillary (100 μm I.D. $\times$ 360 μm O.D) and was then allowed to cure. The end-channel CNT-based amperometric detector offered favorable S/N characteristics at a relatively low potential of +0.8 V (*versus* Ag/AgCl wire) for detecting thiol compounds. Fig. 2 shows the typical electropherograms for (a) homocysteine, (b) cysteine, (c) glutathione, and (d) *N*-acetylcysteine recorded with (A) graphite and (B) CNT composite electrode detectors. By using the CNT-epoxy composite electrode as the detection electrode, the four thiols were separated and detected with well-defined and resolved peaks within 130 s at a separation voltage of +2000 V in a 20 mM phosphate running buffer (pH 7.8). It can be also observed that the current response of the four thiols at the CNT composite electrodes is much higher than that at graphite composite electrode under the same operation conditions. This may be attributed to the faster electron transfer rate on the surface of CNT electrode. Highly linear response is obtained for homocysteine, cysteine, glutathione, and *N*-acetylcysteine over the range of 5–50 μM with detection limits of 0.75, 0.8, 2.9, and 3.3 μM , respectively. Good stability and reproducibility (R.S.D. < 5%) were obtained reflecting the minimal adsorption of thiols at the CNT electrode surface. The new microchip protocol should find a

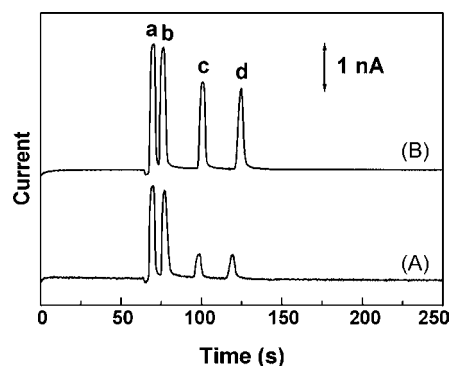


Fig. 2. Electropherograms for mixtures containing 50 μM homocysteine (a), 50 μM cysteine (b), 100 μM glutathione (c), and 100 μM *N*-acetylcysteine (d) at the graphite/epoxy (A) and CNT/epoxy composite (B) microdisc electrodes. Operation conditions: separation and injection voltage, +2000 V; injection time, 2 s; running buffer, 20 mM phosphate buffer (pH 7.8); detector potential, +0.85 V (*vs.* Ag/AgCl wire) (reprinted with permission [34]).

wide range of bioanalytical applications involving assays of thiol compounds.

3. Boron-doped diamond electrochemical detectors in CE

Highly boron-doped diamond films have recently received much attention for electrochemical applications [50–52]. They exhibit very attractive electrochemical properties, such as low, stable background currents [53], a wide potential window in aqueous media [54,55], minimized adsorption of most organic substances [26,56], long-term stability of the response [27,57], and high sensitivity [58,59]. All of these unique properties make boron-doped diamond a promising material, especially for electroanalytical detection. In addition, several outstanding properties of boron-doped diamond itself can be very useful in the extreme conditions of electrochemical detection, including its chemical and physical inertness and the highest known breakdown voltage strength [60].

3.1. Boron-doped diamond electrochemical detectors in microchip CE

Wang's group has fabricated and employed BDD band electrodes as the amperometric detectors of microchip CE system [5]. Chemically vapor-deposited boron-doped diamond films (7 mm $\times$ 0.3 mm $\times$ 40 μm) were glued onto a 33 mm $\times$ 10 mm ceramic plate by quick-setting nonconductive epoxy to fabricate the BDD band electrodes (0.3 mm $\times$ 6.0 mm) that were used for end-column amperometric detection. The favorable performance of the boron-doped diamond electrode was indicated from comparison to a commonly used thick-film carbon detector. The boron-doped diamond electrode offered enhanced sensitivity, lower noise levels, and sharper peaks for several groups of important analytes (nitroaromatic explosives, organophosphate nerve agents, and phenols). The favorable S/N characteristics of the BDD-based CE detector were coupled with a greatly improved resistance to surface fouling and greater isolation from high separation voltages. The enhanced stability was indicated

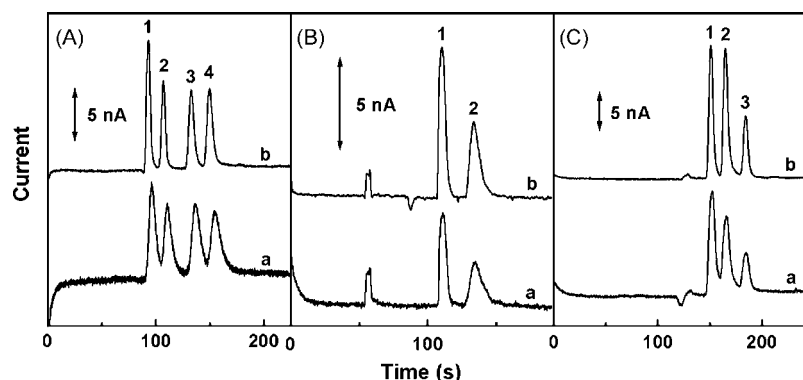


Fig. 3. Electropherograms for phenols (A), nerve agents (B), and explosives (C) with a screen-printed carbon (a) and a boron-doped diamond band (b) electrodes. Sample mixture A: 100 μ M phenol (1), 100 μ M 2-chlorophenol (2), 200 μ M 2,4-dichlorophenol (3), and 200 μ M 2,3-dichlorophenol (3). Sample B: 10 ppm paraoxon (1), 10 ppm methyl parathion (2). Sample C: 15 ppm 1,3-dinitrobenzene, (1), 15 ppm 2,4,6-trinitrotoluene (2), 15 ppm 2,4-dinitrotoluene (3). Operation conditions: separation medium, 10 mM borate/20 mM phosphate buffer (pH 8.0) (A), 20 mM MES (pH 5.0, containing 10 mM SDS) (B); 15 mM borate buffer (pH 9.2, containing 15 mM SDS) (C), separation voltage, +1500 V (A), +2000 (B), +1000 (C); injection voltage: +1500 V (A), +2000 (B), +1000 V (C); injection time, 2 s; detection potential, +0.95 V (A), -0.55 V (B), -0.70 V (C) (vs. Ag/AgCl wire) (reprinted with permission [5]).

from a R.S.D. of 0.8% for 60 repetitive measurements of 5 ppm 2,4,6-trinitrotoluene (*versus* R.S.D. of 10.8% at the thick-film carbon electrode).

Fig. 3 illustrate the representative electropherograms for phenols (A), organophosphate nerve agents (B), and nitroaromatic explosives (C) recorded with the screen-printed carbon (a) and boron-doped diamond band (b) as the detection electrodes. In all cases, the BDD detector displays higher sensitivity and a lower noise level under the same operational conditions. Notice also the flatter baseline current of the BDD detector, particularly during the initial 20 s. The enhanced signal-to-background characteristics of the BDD electrode are coupled to sharper peaks, and hence to enhanced resolution.

The BDD band electrode (0.3 mm $\times$ 6.0 mm) has also been coupled with microchip CE for the separation and detection of several purines and purine-containing compounds [61]. The BDD end-channel amperometric detector offers favorable S/N characteristics at a very high detection potential (+1.3 V) for detecting purine-related compounds. Five purines (guanine, hypoxanthine, guanosine, xanthine, and uric acid) have been separated within 6 min at a separation voltage of +1000 V using a running buffer containing borate and phosphate (pH 8.2). Linear calibration plots were observed for micromolar concentrations of the purine compounds. Good stability and reproducibility (R.S.D. < 5%) are obtained, indicating the minimal adsorption of purines at the BDD surface. The attractive features of the BDD thin-film detector has also been demonstrated by the detection of several dye-related amino-substituted aromatic compounds in connected with the separation of microchip CE [62]. The boron-doped diamond electrode was employed in the end-column amperometric detection of 4-aminophenol (4-AP), 1,2-phenylenediamine (1,2-PDA), 2-aminonaphthalene (2-AN), 2-chloroaniline (2-CA), and *o*-aminobenzoic acid (*o*-ABA). Its attractive behavior was compared to the commonly used screen-printed carbon and glassy-carbon electrodes. These conventional electrode materials exhibit a significant degree of passivation and low sensitivity to the environmental pollutants mentioned above while the boron-doped diamond detection electrode dis-

played a favorable analytical performance, including lower noise levels, higher peak resolution with enhanced sensitivity, and improved resistance against electrode fouling. The boron-doped diamond detector displayed a wide linear response over the 2–50 μ M range with the detection limits of 2.0 and 1.3 μ M for 4-AP and 2-AN, respectively. The enhanced stability was demonstrated by repetitive measurements ($n = 7$) with the R.S.D. values of 1.4% and 4.7% for the peak currents of 100 μ M 1,2-PDA and 200 μ M 2-CA, respectively.

3.2. Boron-doped diamond electrochemical detectors in conventional CE

Boron-doped diamond electrode was firstly coupled with conventional CE by Shin et al. for end-column electrochemical detection [60]. BDD microline electrodes (exposed surface area, 300 μ m $\times$ 50 μ m) were fabricated from conducting boron-doped diamond thin films. Their analytical performance as CE detectors was evaluated in connection with a laboratory-made CE system. The unique system exhibited high separation efficiency for the detection of several catecholamines, including dopamine (DA), norepinephrine (NE), and epinephrine (E), with excellent analytical performance. The number of theoretical plates (N) for DA was determined to be 155,000 plates/m. The boron-doped diamond electrochemical detection system displayed not only low detection limits (~ 20 nM for E at S/N = 3) but also highly reproducible current responses (R.S.D. < 5%) for 10 repetitive analyses of a mixture containing 50 μ M DA, NE, and E. The performance of the boron-doped diamond detector in CE was also evaluated in the detection of chlorinated phenols. Compared with the carbon fiber microelectrode, the boron-doped diamond electrode exhibited lower detection limits resulting from very low noise levels and highly reproducible measurement without electrode polishing due to the low surface fouling, which makes it possible to perform easier and more stable CE analysis.

Swain's group has done a series of work to demonstrate the unique proprieties of the BDD electrodes when they

were employed as the electrochemical detector of conventional CE [63–65]. The microelectrodes were prepared by coating thin films of polycrystalline boron-doped diamond on electrochemically sharpened platinum wires, using microwave-assisted chemical vapor deposition (CVD) [63]. The boron-doped diamond microelectrodes were characterized by scanning electron microscopy, Raman spectroscopy, and cyclic voltammetry. The microelectrodes sealed in polypropylene pipette tips were employed for the end-column detection in CE. Evaluation of the CE-ECD system and the electrode performance were accomplished using dopamine, catechol, and ascorbic acid as test analytes. The background current (~ 100 pA) and noise (~ 3 pA) were measured at different detection potentials and found to be very stable with time. Reproducible separation (migration time) and detection (peak current) of dopamine, catechol, and ascorbic acid were observed with the precisions of 4.1% or less. Calibration curves plotted from the peak area were linear over four orders of magnitude, up to a concentration range between 0.1 and 1 mM. The limits of detection for dopamine and catechol were 1.7 and 2.6 fmol, respectively ($S/N=3$). The separation efficiency was determined to be 33,000, 56,000, and 98,000 plates/m for dopamine, catechol, and ascorbic acid, respectively. The separation and detection of α - and β -naphthol in 160 mM borate buffer (pH 9.2) were also investigated. Separation of these two analytes was achieved with efficiencies of 118,000 and 126,000 plates/m, respectively.

Swain's group has also employed the boron-doped diamond film electrode coated on a sharpened platinum wire for the direct and indirect amperometric detection of the chlorinated phenolic pollutants in connection with conventional CE [64]. The boron-doped diamond microelectrode was used in end-column detection and exhibited a low and stable background current with low peak-to-peak noise and good electrochemical activity for the phenolic pollutants without any conventional pretreatment. The electrode performance was evaluated by the separation and detection of phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol. The typical electropherogram was illustrated in Fig. 4. The seven phenols can be detected at the boron-doped diamond microelectrode to give well-defined and resolved peaks within 900 s with no evidence of any electrode fouling. As an example, the concentration limit of quantitation for 2-chlorophenol was 100 nM or 13 ppb ($S/N=3$) and the R.S.D. of the peak height for nine injections was 4.7%. The separation efficiency was greater than 100,000 plates/m for all the seven analytes. The microelectrode was also employed for the indirect detection of the chlorinated phenols. Satisfactory electropherograms were also achieved for the separation and detection of 2-chlorophenol, 3-chlorophenol, and 2,4-dichlorophenol in this mode, although the linear dynamic range was not as wide and the limit of quantitation was not as low as in direct amperometry. The concentration limit of quantitation for these pollutants was in the middle micromolar range (1–10 ppm) with excellent response reproducibility of 3.2%, or less.

In addition, they have analyzed chlorinated phenols (2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol) in

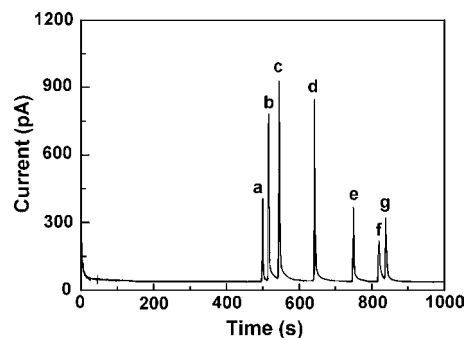


Fig. 4. A typical electropherogram for a mixture containing 20 μ M phenol (a), 4-chlorophenol (b), 3-chlorophenol (c), 2-chlorophenol (d), 2,4-dichlorophenol (e), 2,4,6-trichlorophenol (f), and pentachlorophenol (g) using boron-doped diamond detection electrode. Separation voltage, +20 kV; capillary, 27 μ m i.d. $\times$ 76 cm long; running buffer, 10 mM borate/20 mM phosphate buffer (pH 8.4); detection potential, +1.05 V (vs. Ag/AgCl); injection, 8 kV for 3 s (reprinted with permission [64]).

river water using off-line solid-phase extraction (SPE) and conventional CE coupled with electrochemical detection [65]. A key to the sensitive, reproducible, and stable detection of these pollutants was the use of the BBD microelectrode mentioned above in amperometric detection mode. Off-line SPE was employed to extract and preconcentrate the pollutants prior to CE analysis. Pollutant recoveries in the 95–100% range with R.S.D. of 1–4% were achieved. The boron-doped diamond microelectrode provided a low and stable background current with low peak-to-peak noise. The oxidative detection of the pollutants was accomplished at +1.05 V (*versus* Ag/AgCl) without the need for electrode pretreatment. A reproducible electrode response was observed during multiple injections of the chlorinated phenol solutions with a R.S.D. of less than 5.4%. Good electrode response stability was observed over many days of continuous use with no significant electrode deactivation or fouling. The minimum concentration detectable for all the six phenolic pollutants ranged from 0.02 to 0.2 ppb ($S/N \geq 3$) using a 250:1 preconcentration factor.

4. Conclusions

In summary, both CNT and boron-doped diamond are promising materials for fabricating the electrochemical detectors of microchip and conventional CE systems due to their unique attributes. The CNT-based detectors in connection with microchip and conventional CE can be classified into surface modified, paste, and solid composite electrodes. All these CNT-based CE detectors offer favorable signal-to-background characteristics, good resistance to surface fouling, strong electrocatalytic activity, sharp peaks for the analytes, and simple design and fabrication. The attractive properties of BDD electrodes include their favorable signal-to-background characteristics, negligible adsorption of organic compounds, and wide potential window that make the BDD a very promising material for the electrochemical detection in microchip and conventional CE systems. Boron-doped diamond electrodes facilitate the detection of analytes that possess high redox potentials and display surface fouling effects. It can be concluded that microchip

and conventional CE with CNT or boron-doped diamond detection electrodes should find a wide range of application in the field of biomedical and pharmaceutical analysis, clinical diagnostics, environmental monitoring, etc.

Congratulations

I would like to take this opportunity to congratulate Dr. Wang on the occasion of his 60th birthday. He is one of the most pioneering electroanalyst with wonderful accomplishments.



Acknowledgments

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References

- [1] J.W. Jorgenson, K.D. Lukacs, *J. Chromatogr.* 218 (1981) 209.
- [2] J.W. Jorgenson, K.D. Lukacs, *Anal. Chem.* 53 (1981) 1298.
- [3] D.R. Reyes, D. Iossifidis, P.A. Auroux, A. Manz, *Anal. Chem.* 74 (2002) 2623.
- [4] P.A. Auroux, D. Iossifidis, D.R. Reyes, A. Manz, *Anal. Chem.* 74 (2002) 2637.
- [5] J. Wang, G. Chen, M.P. Chatrathi, A. Fujishima, D.A. Tryk, D. Shin, *Anal. Chem.* 75 (2003) 935.
- [6] M. Pumera, *Talanta* 66 (2005) 1048.
- [7] W.R. Vandaveer, S.A. Pasas, R.S. Martin, S.M. Lunte, *Electrophoresis* 23 (2002) 3667.
- [8] J. Wang, *Talanta* 56 (2002) 223.
- [9] N.A. Lacher, K.E. Garrison, R.S. Martin, S.M. Lunte, *Electrophoresis* 22 (2001) 2526.
- [10] J. Wang, M.P. Chatrathi, B.M. Tian, *Anal. Chim. Acta* 416 (2000) 9.
- [11] R.S. Martin, K.L. Ratzlaff, B.H. Huynh, S.M. Lunte, *Anal. Chem.* 74 (2002) 1136.
- [12] W.R. Vandaveer, S.A. Pasas-Farmer, D.J. Fischer, C.N. Frankenfeld, S.M. Lunte, *Electrophoresis* 25 (2004) 3528.
- [13] L. Nyholm, *Analyst* 130 (2005) 599.
- [14] S. Iijima, *Nature* 354 (1991) 56.
- [15] R.H. Baughman, A. Zakhidov, W.A. de Heer, *Science* 297 (2002) 787.
- [16] P.M. Ajayan, *Chem. Rev.* 99 (1999) 1787.
- [17] J. Wang, *Electroanalysis* 17 (2005) 7.
- [18] R.S. Chen, W.H. Huang, H. Tong, Z.L. Wang, J.K. Cheng, *Anal. Chem.* 75 (2003) 6341.
- [19] C.E. Banks, T.J. Davies, G.G. Wildgoose, R.G. Compton, *Chem. Commun.* (7) (2005) 829.
- [20] Z.H. Wang, J. Liu, Q.L. Liang, Y.M. Wang, G. Luo, *Analyst* 127 (2002) 653.
- [21] G. Zhao, S.Q. Zang, K.Z. Liu, S. Lin, J. Liang, X.Y. Guo, Z.J. Zhang, *Anal. Lett.* 35 (2002) 2233.
- [22] J. Wang, M. Musameh, *Analyst* 128 (2003) 1382.
- [23] M. Musameh, J. Wang, A. Merkoci, Y.H. Lin, *Electrochem. Commun.* 4 (2002) 743.
- [24] C. Prado, G.U. Flechsig, P. Grundler, J.S. Foord, F. Marken, R. Compton, *Analyst* 127 (2002) 329.
- [25] N. Spataru, B.V. Sarada, E. Popa, D.A. Tryk, A. Fujishima, *Anal. Chem.* 73 (2001) 514.
- [26] J. Xu, G.A. Swain, *Anal. Chem.* 70 (1998) 1502.
- [27] T.N. Rao, I. Yagi, T. Miwa, D.A. Tryk, A. Fujishima, *Anal. Chem.* 71 (1999) 2506.
- [28] C. Terashima, N. Rao Tata, B.V. Sarada, D.A. Tryk, A. Fujishima, *Anal. Chem.* 74 (2002) 895.
- [29] F. Marken, C.A. Paddon, D. Asogan, *Electrochem. Commun.* 4 (2002) 62.
- [30] J. Wang, G. Chen, M.P. Chatrathi, M. Musameh, *Anal. Chem.* 76 (2004) 298.
- [31] S. Qu, J. Wang, J.L. Kong, P.Y. Yang, G. Chen, *Talanta* 71 (2007) 1096.
- [32] J. Wang, M. Musameh, *Anal. Chem.* 75 (2003) 2075.
- [33] J. Wang, M. Musameh, *Analyst* 129 (2004) 1.
- [34] G. Chen, L.Y. Zhang, W. Wang, *Talanta* 64 (2004) 1018.
- [35] J. Wang, G. Chen, M. Wang, M.P. Chatrathi, *Analyst* 129 (2004) 512.
- [36] H. Luo, Z. Shi, N. Li, Z. Gu, Q. Zhuang, *Anal. Chem.* 73 (2001) 915.
- [37] F.H. Wu, G.C. Zhao, X.W. Wei, *Electrochem. Commun.* 4 (2002) 690.
- [38] J. Wang, M. Musameh, Y. Lin, *J. Am. Chem. Soc.* 125 (2003) 2408.
- [39] M. Zhang, A. Smith, W. Gorski, *Anal. Chem.* 76 (2004) 5045.
- [40] X.L. Luo, J.J. Xu, J.L. Wang, H.Y. Chen, *Chem. Commun.* 16 (2005) 2169.
- [41] M.L. Guo, J.H. Chen, J. Li, B. Tao, S.Z. Yao, *Anal. Chim. Acta* 532 (2005) 71.
- [42] M. Gao, S. Huang, L. Dai, G. Wallace, R. Gao, Z. Wang, *Angew. Chem. Int. Ed.* 39 (2000) 3664.
- [43] M. Pumera, X. Llopis, A. Merkoci, S. Alegret, *Microchim. Acta* 152 (2006) 261.
- [44] M. Vlckova, M.A. Schwarz, *J. Chromatogr. A* 1142 (2007) 214.
- [45] X. Yao, H.X. Wu, J. Wang, S. Qu, G. Chen, *Chem. Eur. J.* 13 (2007) 846.
- [46] M. Chicharro, A. Sanchez, E. Bermejo, A. Zapardiel, M.D. Rubianes, G.A. Rivas, *Anal. Chim. Acta* 543 (2005) 84.
- [47] M. Pumera, A. Merkoci, S. Alegret, *Electrophoresis* 28 (2007) 1274.
- [48] M. Pumera, A. Merkoci, S. Alegret, *Electrophoresis* 27 (2006) 5068.
- [49] X. Yao, X.J. Xu, P.Y. Yang, G. Chen, *Electrophoresis* 27 (2006) 3233.
- [50] G.M. Swain, A.B. Anderson, J.C. Angus, *MRS Bull.* 9 (1998) 56.
- [51] Y.V. Pleskov, Y.E. Evstefeeva, M.D. Kvotova, V. Laptev, *Electrochim. Acta* 44 (1999) 3361.
- [52] T.N. Rao, A. Fujishima, *Diamond Relat. Mater.* 9 (2000) 384.
- [53] Y. Yano, D.A. Tryk, K. Hashimoto, A. Fujishima, *J. Electrochem. Soc.* 145 (1998) 1870.
- [54] J.W. Strojek, M.C. Granger, T. Dallas, M.W. Holtz, G.M. Swain, *Anal. Chem.* 68 (1996) 2031.
- [55] N. Vinokur, B. Miller, Y. Avyigal, R. Kalish, *J. Electrochem. Soc.* 143 (1996) L238.
- [56] A. Fujishima, T.N. Rao, E. Popa, I. Yagi, D.A. Tryk, *J. Electroanal. Chem.* 473 (1999) 179.
- [57] E. Popa, H. Notsu, T. Miwa, D.A. Tryk, A. Fujishima, *Electrochem. Solid-State Lett.* 2 (1999) 49.
- [58] C. Terashima, T.N. Rao, B.V. Sarada, D.A. Tryk, A. Fujishima, *Anal. Chem.* 74 (2002) 895.
- [59] T.N. Rao, B.H. Loo, B.V. Sarada, C. Terashima, A. Fujishima, *Anal. Chem.* 74 (2002) 1578.
- [60] D.C. Shin, B.V. Sarada, D.A. Tryk, A. Fujishima, *Anal. Chem.* 75 (2003) 530.
- [61] J. Wang, G. Chen, A. Muck, D.C. Shin, A. Fujishima, *J. Chromatogr. A* 1022 (2004) 207.
- [62] D.C. Shin, D.A. Tryk, A. Fujishima, A. Muck, G. Chen, J. Wang, *Electrophoresis* 25 (2004) 3017.
- [63] J. Cvacka, V. Quaiserova, J. Park, Y. Show, A. Muck, G.M. Swain, *Anal. Chem.* 75 (2003) 2678.
- [64] G.W. Muna, V. Quaiserova-Mocko, G.M. Swain, *Electroanalysis* 17 (2005) 1160.
- [65] G.W. Muna, V. Quaiserova-Mocko, G.M. Swain, *Anal. Chem.* 77 (2005) 6542.

Comparative study of multi walled carbon nanotubes-based electrodes in micellar media and their application to micellar electrokinetic capillary chromatography

Manuel Chicharro^{a,*}, Alberto Sánchez Arribas^{a,*}, Mónica Moreno^a,
Esperanza Bermejo^a, Antonio Zapardiel^b

^a Departamento de Química Analítica y Análisis Instrumental, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain

^b Departamento de Ciencias Analíticas, Facultad de Ciencias, Universidad Nacional de Educación a Distancia, 28040 Madrid, Spain

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Abstract

This work reports on a comparative study of the electrochemical performance of carbon nanotubes-based electrodes in micellar media and their application for amperometric detection in micellar electrokinetic capillary chromatography (MEKC) separations. These electrodes were prepared in two different ways: immobilization of a layer of carbon nanotubes dispersed in polyethylenimine (PEI), ethanol or Nafion onto glassy carbon electrodes or preparation of paste electrodes using mineral oil as binder. Scanning electron microscopy (SEM) was employed for surface morphology characterization while cyclic voltammetry of background electrolyte was used for capacitance estimation. The amperometric responses to hydrogen peroxide, amitrol, diuron and 2,3-dichlorophenol (2,3CP) in the presence and in the absence of sodium dodecylsulphate (SDS) were studied by flow injection analysis (FIA), demonstrating that the electrocatalytic activity, background current and electroanalytical performance were strongly dependent on the electrodes preparation procedure. Glassy carbon electrodes modified with carbon nanotubes dispersed in PEI (GC/(CNT/PEI)) displayed the most adequate performance in micellar media, maintaining good electrocatalytic properties combined with acceptable background currents and resistance to passivation. The advantages of using GC/(CNT/PEI) as detectors in capillary electrophoresis were illustrated for the MEKC separations of phenolic pollutants (phenol, 3-chlorophenol, 2,3-dichlorophenol and 4-nitrophenol) and herbicides (amitrol, asulam, diuron, fenuron, monuron and chlortoluron).

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1. Introduction

Carbon nanotubes (CNT), discovered by Ijima [1] represent a new type of carbon material with unique electronic, mechanical and structural properties that depend on their geometries and dimensions [1–3]. CNTs consist of cylinders of graphite layers with both ends capped with half fullerene [3]. This nanostructured material has received enormous attention in electrochemistry mainly due to their electrocatalytic effects and

ability to promote electron transfer reactions, which has been ascribed to the presence of defects in the graphite structure, both at the ends or the side walls, which break the carbon bonds and improve in these sites the electron transfer kinetic [4].

The electrochemical activity of CNT is strongly dependent on their fabrication process, intrinsic structure, occluded metal impurities, dispersion procedure and electrode preparation [5]. Usual fabrication of carbon nanotubes is carried out by chemical vapour deposition (CVD) or arc discharge (ARC) processes and the properties and structure of the produced CNT is substantially different. CVD route employs metal catalyst, whose size and quantity determine the diameter and the wall thickness of CNT, and the final product usually contains certain amount of metal nanoparticles occluded within their walls and the density of

* Corresponding authors.

E-mail addresses: manuel.chicharro@uam.es (M. Chicharro),
alberto.sanchez@uam.es (A.S. Arribas).

structural defects is relatively high leading to enhanced electrochemical properties [5]. On the opposite, ARC-produced CNT are closed at either ends and they are predominantly straight with a low density of defects that results in not as good electrochemical performance [5]. CNT are formed in two principal types: single-walled carbon nanotubes (SWCNT), which consist of a single tube of graphite, and multiwalled carbon nanotubes (MWCNT) which consist of several concentric tubes of graphite fitted one inside the other. In the case of MWCNT there are a number of morphological variations possible depending on the fabrication conditions and they can be formed as “double-wall”, “hollow tube”, “herringbone” or “bamboo-like”. “Herringbone” and “bamboo-like” possess higher density of defects and their electrochemical performance is usually better [6]. Before using and in order to remove residual amorphous carbon and metal impurities, CNT are often subjected to purification treatments that include “washing” in diluted or concentrated acids during long periods (24–72 h) assisted by ultrasonic exposure [7–9]. These treatments sometimes do not modified the electrochemical properties of CNT [10] but many times have supposed a dramatic enhance of these properties, specially for ARC-produced CNT, due to oxidative functionalization (introduction of carboxylic groups), rupture of carbon bonds (hence introduction of defects) or cutting in smaller pieces [5,11]. Some recent studies have also reported that such acidic treatments are not able to remove completely the metal impurities [5,8,9], since they are protected in the core of the nanotube walls and remain electroactive [9]. In addition, there are some examples where the observed electrocatalytic effect of carbon nanotubes is actually due to the presence of residual catalyst nanoparticles (mainly iron oxides) occluded within the walls [12–14].

The integration of CNT in devices for developing electrochemical sensors has been carried out mainly by their incorporation in composites, using mineral oil [15,16], Teflon [17] or epoxy composites [18] as binders, or by the modification of conventional electrode surfaces. Important examples of ordered electrode modification could be found in the literature [19], but the random modification of electrodes' surface by CNT films is the most common strategy used in the development of CNT-based electrochemical devices. The insolubility of CNT in common solvents is a problem in these applications and dispersions in solvents such as concentrated sulfuric or nitric acid, *N,N'*-dimethylformamide (DMF), dimethyl sulphonyl oxide (DMSO) or tetrahydrofuran (THF) have been described [5,11,20]. The covalent or non covalent (wrapping) bonding of polymer molecules to CNT have been proved a very successful alternative to solve this problem and Nafion [21,22], Chitosan [23,24], polyvinylpyrrolidone [25], polystyrene sulfonate [25] or polyethylenimine [26] have been employed. Anyway the electrochemical performance of the CNT-modified electrode will be strongly influenced by the dispersant nature and the dispersion procedure.

The integration of carbon nanotube-based electrochemical detector in micro-fluidic separation systems is a promising alternative to circumvent their traditional sensitivity problems that arise from the extremely low volume samples introduced. Electrochemical detection, mainly in the amperometry mode, full fits

the miniaturization requirements of capillary electrophoresis by simple and relatively inexpensive arrangements and without the loss of sensitivity observed in other detection modes [27]. The coupling of CNT-based sensors to microfluidic systems was first developed by Prof. Wang's group in 2004 for the analysis of purines, chlorophenols, hydrazines and neurotransmitters [28]. Despite of the advantages shown in this seminal work, the use of CNT-based detectors is spreading slowly and still scarce applications to the analysis of neurotransmitters [29–31], carbohydrates [32], aminoacids [32,33], thiols [34] and natural [35,36] and pollutant [37] phenolic compounds have been reported. In most of them, the free zone capillary electrophoresis mode (CZE) was employed for the separation and it means that analytes must be charged to be resolved. The separation of neutral analytes by capillary electrophoresis could be achieved using the micellar electrokinetic capillary chromatography (MEKC) mode. This electrophoretic mode is based in the use of separation buffers containing surfactants (typically sodium dodecyl sulphate (SDS) or cetyl trimethyl ammonium salts) where the analytes are in a dynamic distribution equilibrium between the hydrophobic micelle core and the bulk buffer solution. This has extended the versatility of electrophoretic separations to important analytes in biomedical and pharmaceutical analysis, clinical diagnostics, forensic investigations or environmental monitoring [38–40]. The application of electrochemical detection in connection with MEKC is subjected to the micellar effects in the electrochemistry, as adsorption of surfactants on electrodes may influence the redox potential as well the stability of the electrogenerated products [41,42].

In this article, we address the electrochemical performance of multiwalled carbon nanotubes modified electrodes in micellar environments focused to their analytical application as amperometric detector in MEKC separations, solely reported to date for a neurotransmitters metabolites analysis [30]. The capabilities of the CNT-based detectors are illustrated for the separations of phenolic contaminants (phenol, 3-chlorophenol, 2,3-dichlorophenol and 4-nitrophenol) and herbicides (amitrol, asulam, diuron, monuron, chlortoluron and fenuron).

2. Experimental

2.1. Reagents

Hydrogen peroxide (30%, v/v aqueous solution) was purchased from Scharlau (Barcelona, Spain). Potassium hexacyanoferrate (III), hexaammineruthenium (III) chloride, 1,4-benzoquinone, phenol, 3-chlorophenol, 2,3-dichlorophenol, 4-nitrophenol and polyethylenimine (PEI; Cat. no. P3143) were purchased from Sigma (Madrid, Spain). Fenuron, monuron, chlortoluron, diuron and asulam were obtained from Riedel-de-Haën (Madrid, Spain). 3-Amino-1,2,4, triazole (amitrol) was from Fluka (Madrid, Spain). Sodium dodecyl sulphate (SDS) and Nafion (Cat. no. 527084) were from Aldrich (Madrid, Spain). Multiwalled carbon nanotubes powder (MWCNT, 30 ± 15 nm diameter, 5–20 μ m length, purity >95%, “hollow tube” type, prepared by chemical vapour deposition, nominal 1% Fe and 0.1% S residuals, Lot. PD30L520-60805) were obtained

from NanoLab (USA). All other chemicals were analytical grade and were used as received without any further purification. Ultrapure water ($\rho > 18 \text{ M}\Omega \text{ cm}$) from a Millipore-MilliQ system was used for preparing all aqueous solutions. Stock solutions of hydrogen peroxide, amitrol, phenol, 3-chlorophenol, 2,3-dichlorophenol and 4-nitrophenol were prepared in water while asulam, fenuron, monuron, chlortoluron and diuron were dissolved in methanol. These solutions were kept away from light and stored in the fridge before using. Diluted solutions of these analytes in the corresponding buffers were prepared daily. Buffers and samples for capillary electrophoresis were sonicated for 5 min and filtered through $0.45 \mu\text{m}$ MFS-13 filters (Advantec MFS, Inc., USA).

2.2. Apparatus

Scanning electron microscopy (SEM) images were obtained a Hitachi S-3000N electron microscope using an accelerating voltage of 30 kV. Samples were placed in aluminum stubs and were then coated with a gold layer by sputtering in order to enhance their conductivity.

Cyclic voltammetry (CV) experiments were conducted in a 10 mL cell using the conventional three electrode configuration. Bare glassy carbon electrodes (GC, 3 mm diameter, from CHI, Austin, USA), CNT dispersion-modified GC and graphite or CNT paste electrodes (3 mm diameter) were employed as working electrode and a Ag/AgCl 3 M NaCl (BAS, West Lafayette, USA) and a Pt wire were used as reference and counter electrode, respectively. The voltammetric measurements were performed with a BAS 100B electrochemical analyzer (BAS, West Lafayette, USA) connected to a Pentium IV PC computer with BAS W 2.3 software (BAS, West Lafayette, USA) incorporated for data storage and conversion to “txt” files.

Flow injection (FIA) experiments were carried out with a peristaltic pump (ISMATEC, ISM 834) connected to a four way loop pressure valve Rheodyne type 5020 (Altech, Spain) with a $100 \mu\text{L}$ sample loop. The connections were made with an interconnecting PTFE tubing (0.8 mm i.d. and 1.6 mm o.d.). The amperometric measurements were performed with a BAS 100B electrochemical analyzer (BAS, West Lafayette, USA), as above described. The electrodes were inserted into a wall-jet cell (EA-1096 Metrohm) containing a gold wire as counter electrode and a Ag/AgCl, 3 M NaCl (Metrohm) as reference electrode. The working electrodes were made of glassy carbon (GC, 3 mm diameter, from CHI, Austin, USA), bare or modified with the CNT's dispersions, and graphite or CNT pastes (3 mm diameter). All experiments were carried out at room temperature by applying the desired operating potential. Currents were allowed to reach a stable baseline prior to the amperometric monitoring.

Capillary electrophoresis experiments were performed with a Spectra PHORESIS 100 (Thermo Quest Corporation, Spain). No variations were introduced in the original commercial set up of the capillary electrophoresis equipment. Amperometric detection was performed with an amperometric detector LC-4CE (BAS, West Lafayette, USA) connected to a Pentium II PC computer with Chrom Card software (Thermo Quest Corpora-

tion, Spain) for data acquisition and processing. A 60 cm fused silica column ($75 \mu\text{m}$ i.d. and $365 \mu\text{m}$ o.d., Teknokroma, Spain) was used with a 2 cm Nafion decoupler for high voltage isolation. The electrochemical device for coupling to commercially available capillary electrophoresis equipments has been previously described [29]. Basically it consists of a Plexiglas ground vial that holds the decoupler and the electrochemical cell which could allocate the working electrode. Silver and platinum wires act as pseudo-reference and counter electrode, respectively.

2.3. Preparation of the working electrodes

2.3.1. Preparation of MWCNT dispersions

They were obtained by dispersing 1.0 mg of MWCNTs within 1.0 mL of the dispersant solution followed by sonication for 15 min. The different dispersants were 1.0 mg/mL PEI solution (prepared in 50:50, v/v ethanol/water), 0.5% Nafion solution (prepared in 50:50, v/v ethanol/water) and a 50:50 (v/v) ethanol/water solution. Five different dispersions from each dispersant solution were prepared and were employed indistinctly in all the experiments.

2.3.2. Preparation of the glassy carbon electrode modified with the dispersions of MWCNT for CV and FIA experiments

The glassy carbon electrode (GC) was first polished using alumina of 0.30 and $0.05 \mu\text{m}$ for 1 min each and cleaned by sonication in water for 30 s. The electrode was subsequently washed with pure water and then modified with the dispersion of MWCNT in the following way: an aliquot of $20 \mu\text{L}$ was dropped on top of the cleaned GCE and allowed to dry for 90 min.

2.3.3. Preparation of MWCNT (CNTPE) and carbon paste (CPE) electrodes

CNTPE were prepared by mixing in an agatha mortar MWCNT powder and mineral oil (Aldrich, Madrid, Spain) in a 50:50 (w/w) ratio during 30 min. CPE were prepared in the same way using graphite powder (Acheson #38, 1–2 μm , Fisher Scientific, Madrid, Spain) and mineral oil in 70:30 (w/w) ratio. A portion of the resulting pastes were packed firmly into the cavity (3.0 mm diameter) of a Teflon tube. The electric contact was done using a stainless steel screw. A new surface was obtained by smoothing the electrode onto weighing paper.

2.3.4. Preparation of glassy carbon electrodes for capillary electrophoresis

These electrodes were constructed by fixing a 1 mm diameter and 5 mm length glassy carbon rod (Goodfellow, UK) with non-conductive cyanoacrilate glue to one side of a conventional FIA nut. The other side was filled with carbon paste (prepared by mixing graphite powder with mineral oil in a 70:30 (w/w) proportion) and the final electrical contact was established with a stainless steel screw. The exposed glassy carbon disk surface was first polished using alumina of 0.30 and $0.05 \mu\text{m}$ for 1 min each and cleaned by sonication in water for 30 s. The electrode was subsequently washed with pure water and then modified with $2.0 \mu\text{L}$ of the MWCNT dispersion as described above.

2.4. Procedures

The amperometric measurements in FIA were conducted in 50 mM phosphate buffer solution at pH 7.40 in the presence or absence of surfactants, by applying a given potential and allowing the transient currents to decay to a steady-state value prior to the addition of the analyte and subsequent current monitoring. All measurements were performed at room temperature.

The electrophoretic separations were carried out at room temperature (22 °C) (column temperature could not be controlled in this CE equipment) while the detecting currents were allowed to reach a stable baseline prior to the amperometric monitoring. At the beginning of each day, the capillary was conditioned by successive flushing with 1.0 M NaOH, 0.10 M NaOH and separation buffer (5 min each). Between runs, the capillary was rinsed consecutively with water, 0.1 M NaOH, water and separation buffer for 3 min. Samples were introduced by hydrodynamic mode for 1.5 s at 6.5 PSI (the injected volume being 79 nL). Pesticides separations were conducted at 20.0 kV (35 μ A) in 10 mM phosphate buffer pH 7.4 with 20 mM SDS. 50 mM phosphate buffer pH 7.4 was placed in the electrochemical cell in all cases. The gap distance between the working electrode and the capillary outlet was fixed at 50 μ m.

3. Results and discussion

As it has been explained in Section 1, the final characteristics of CNT-based electrodes are strongly dependent on the CNT structure, dispersion procedure and integration on the sensing device. In this comparative study we have selected two preparative schemes successfully employed elsewhere for the fabrication of CNT-based electrodes: the modification of a GC surface with a thin film of a CNT dispersion [21,22,26] and the aggregation of CNT within mineral oil in order to obtain a paste [15,16]. For the former scheme, three different dispersing solutions containing each one the cationic polymer polyethylenimine (PEI) (1.0 mg mL⁻¹ in ethanol/water 1:1), the negatively charged perfluorosulfonated polymer Nafion (0.5% in ethanol/water 1:1) and ethanol (50% in water) were employed. Comparative characterization studies of these CNT-based electrodes are described in the following sections.

3.1. Microscopy imaging characterization

The surface study of GC electrodes modified with different CNT dispersions was carried out by scanning electron microscopy (SEM) at an acceleration voltage of 30 kV and typical images of these electrodes obtained at different magnifications are shown in Fig. 1. In an early view, it is clear that the presence of polymers in the dispersing media resulted in more uniform deposits and a higher roughness was observed at ethanol-dispersed CNT-modified electrodes. The inspection of 20,000 $\times$ magnification images (Fig. 1A, C and E) reveals a similar framework of interwoven CNT, with a close contact between them, for the three different electrodes, with some superposed CNT platforms in the ethanol dispersion. The general surfaces view at 500 $\times$ (Fig. 1B, D and F) shows that the CNT/PEI layer

covered uniformly the whole electrode surface while some discovered islets could be found in the other (see the black islands in the Fig. 1B and D). This observation suggests that electrodes modified with CNT/PEI dispersion display a higher coverage and more CNT active surface is available when they are compared to those obtained with CNT/nafion dispersion.

3.2. Capacitance and surface active estimation by cyclic voltammetry

The structure and the charge distribution of the active layers and surfaces of the selected CNT-based electrodes are very different and this could affect the structure and behavior of the double layer when the electrodes are polarized. In this sense, the contribution of the double layer was evaluated by the estimation of capacitances of the studied electrodes, obtained from cyclic voltammograms at various scan rates (10–200 mV s⁻¹) carried out in 0.050 M phosphate pH 7.40 buffer, in the presence and in the absence of 20 mM SDS.

The profiles of the cyclic voltammograms of GC electrodes modified with CNT dispersions showed flat and symmetric background currents in the whole potential range studied (–0.200 to +0.800 V) indicating that the capacitive current is non-dependant on the potential (not shown). These background currents increased proportionally with the scan rate and capacitances were calculated directly from the slope of the currents recorded at +0.400 V against the scan rate [43]. These calculated capacitances are resumed in Table 1. In the presence of 20 mM SDS, a slight increase in the background current was noticed, pointing that the surfactant had a weak interaction with the CNT layer. On the other hand, the background currents at bare GC electrodes were 100 lower with a more asymmetric profile, the last probably as a result of Faradaic contributions of some carboxyl and quinone groups confined in the glassy carbon surface. In addition, the presence of 20 mM SDS in the background electrolyte increased the voltammetric currents twice. The comparison of this increase with those observed at CNT-modified GC shows that SDS had a stronger interaction with glassy carbon and it was incorporated within the electrode surface, probably by adsorption of the hydrophobic alkyl chain, leaving the negatively charged sulfate groups exposed to the bulk solution so a higher charge density contributed to the capacitive current.

In the experiments carried out using paste electrodes, the background currents in phosphate buffer at CNTPE were about 300 times lower than at CNT-modified GC and the half of plain CPE and bare GC (not shown). This effect is ascribed to the different morphology of electrodes, where CNT are embedded within the mineral oil binder in CNTPE [29,44] and part of their active sites are impeded by the hydrophobic layer while the dispersed CNT cast onto GC seemed to be more accessible. The contact of this hydrophobic mineral oil layer with SDS supposed a dramatic increase in the background current at both CNTPE (about 300 times) and CPE (about 130 times) due to a massive incorporation of the surfactant to the electrodes surface which largely increased their charge density.

For a better understanding of the capacitance results obtained in these experiments, their normalization to specific capaci-

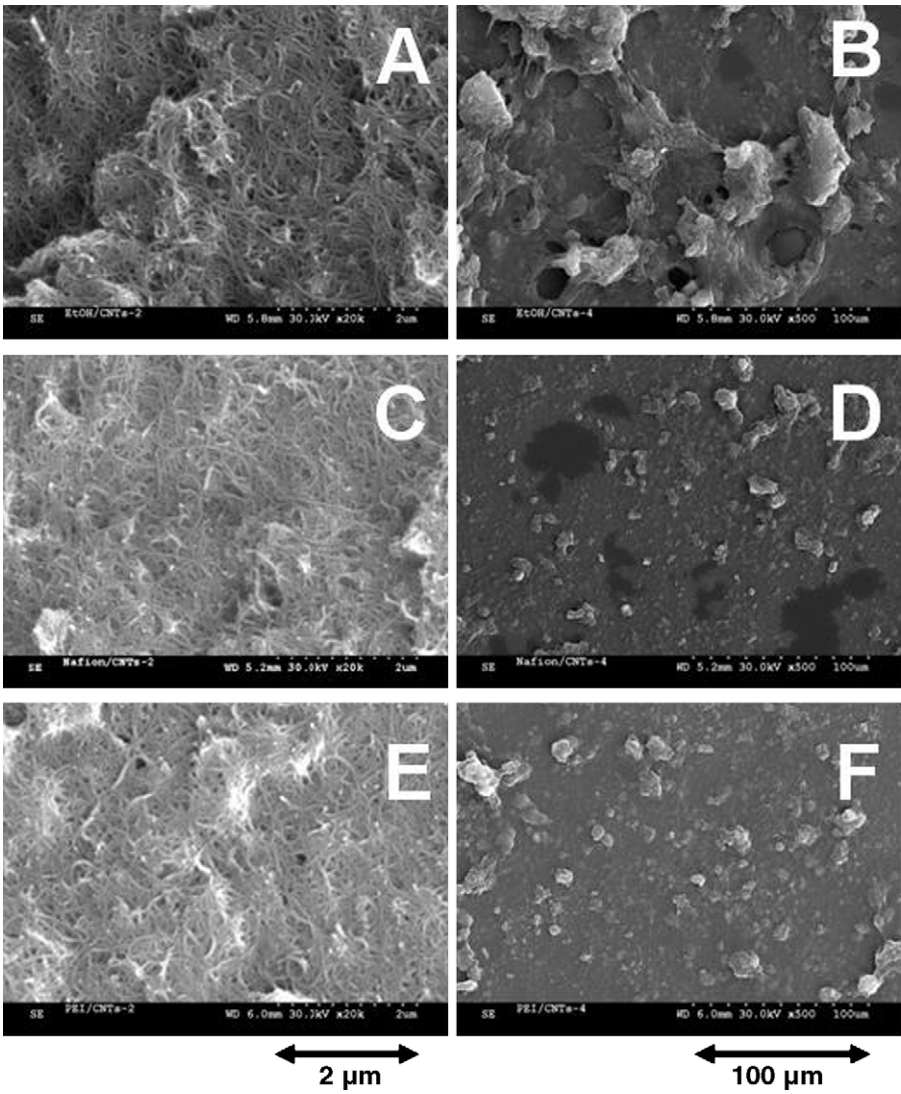


Fig. 1. Scanning electron micrographs of CNT/EtOH (A and B), CNT/Nafion (C and D) and CNT/PEI (E and F) dispersions onto GC surfaces. Acceleration voltage, 30 kV. Magnification, 20,000× (A, C and E) and 500× (B, D and F).

Table 1
Capacitances and effective active surface areas

		Capacitance (μF) ^a	Active area (cm ²) ^b	Specific capacitance (μF cm ⁻²)
GC	PBS ^c	2.10	0.0634	33.1
	SDS ^d	4.21		66.4
GC/(CNT/PEI)	PBS ^c	327	0.736	444
	SDS ^d	368		500
GC/(CNT/EtOH)	PBS ^c	380	0.760	500
	SDS ^d	438		576
GC/(CNT/Nafion)	PBS ^c	271	0.430	630
	SDS ^d	306		712
CPE	PBS ^c	2.52	0.0789	31.9
	SDS ^d	303		3840
CNTPE	PBS ^c	1.14	0.0811	14.0
	SDS ^d	321		3958

^a Slope of the voltammetric currents recorded at +0.400 V against the scan rate.
^b Obtained from the slopes of *i*_p vs. *v*^{1/2} of cyclic voltammograms of 0.80 mM 1,4-benzoquinone in 0.050 M phosphate buffer pH 7.40.
^c 0.050 M phosphate buffer pH 7.40.
^d 0.050 M phosphate buffer pH 7.40 + 20 mM SDS.

tance was necessary and then the effective active surface of the different electrodes was calculated. Cyclic voltammograms of 0.80 mM 1,4-benzoquinone in 0.050 M phosphate buffer pH 7.4 were carried out from 10 to 255 mV s⁻¹ and surface areas were calculated from the slope of the cathodic peak current against the square root of the scan rate using the Randles–Sevcik equation:

$$i_p = (2.69 \times 10^5) AD^{1/2} n^{3/2} C^* v^{1/2} \quad (1)$$

where i_p is the voltammetric peak current, A the effective active surface area, D the diffusion coefficient, n the number of electrons, C^* the bulk concentration of the electroactive substance and v is the scan rate.

1,4-Benzoquinone was selected since it is a neutral compound under the measuring conditions and the possible disturbances in the diffusion controlled electrochemical process created by the electrostatic interactions of charged dispersant polymers with charged targets, such as ferricyanide, are avoided. Its electrochemical reduction in aqueous buffer to 1,4-hydroquinone involving 2 electrons and 2 protons is well known and the diffusion coefficient employed in these calculations was 4.60×10^{-6} cm² s⁻¹ [45].

Cyclic voltammograms at all tested electrodes showed well-defined reduction waves in the +0.050 V to -0.150 V range whose peaks current followed a linear relationship with the square root of scan rate, thus the electrochemical process was diffusion controlled. Surface active areas were calculated by applying Eq. (1) and they are summarized in Table 1. GC electrodes modified with CNT dispersions presented larger area, from 5 to 10 times, than bare GC while CNTPE and CPE had similar values. This result is in accordance with the conclusions of microscopic imaging experiments, where a significant surface roughness was observed at GC modified with CNT dispersions and a lower coverage of GC surface was noticed when using CNT/naion dispersion. Active surface areas of GC, CNTPE and CPE were closer to their geometric area, 0.071 cm². The calculated CNTPE area reflects that CNT are covered by the mineral oil binder and their active sites are less exposed to the solution than at the electrodes modified with CNT dispersions.

The specific capacitances were calculated and they are resumed in Table 1. The corrected values show that GC modified with CNT dispersions had about 15–20 times higher specific capacitances than bare GC in good agreement with Lawrence et al studies with MWCNT dispersions [5] and Pumera studies with single-wall and double-wall CNT dispersions [46]. In addition, CNT/Nafion modified electrodes had higher specific capacitance than CNT/EtOH and CNT/PEI suggesting higher coverage of CNT walls by Nafion polymer and a certain excess of negative charge within CNT surface that it is partially neutralized by the polycation PEI, resulting in a decrease in the capacitive contribution. This effect is also observed in the presence of SDS, where the specific capacitance had larger increase at CNT/Nafion than at CNT/EtOH and CNT/PEI suggesting weak interaction with the surfactant and some charge neutralization between cationic PEI and the incorporated anionic SDS molecules.

Table 2

Cyclic voltammetry of 1.0 mM potassium hexacyanoferrate (III) in 0.1 M KCl

	i_p vs. $v^{1/2a}$	$\log i_p$ vs. $\log v^a$	ΔE_p vs. v^b
GC	$0.7 - 1.42v^{1/2}$	$0.21 + 0.48\log v$	$75 + 0.096v$
GC/(CNT/PEI)	–	$0.61 + 0.77\log v$	$59 + 0.71v$
GC/(CNT/EtOH)	$-5 - 5.71v^{1/2}$	$0.50 + 0.61\log v$	$52 + 0.17v$
GC/(CNT/Nafion)	$2.2 - 1.60v^{1/2}$	$0.45 + 0.40\log v$	$108 + 0.13v$
CPE	$1.4 - 0.92v^{1/2}$	$0.20 + 0.41\log v$	$370 + 0.59v$
CNTPE	$1.7 - 1.14v^{1/2}$	$0.28 + 0.42\log v$	$204 + 0.54v$

^a i_p were in μA and v in mV s⁻¹.

^b ΔE_p were in mV and v in mV s⁻¹.

3.3. Characterization of the voltammetric response to charged analytes

It has been shown in the precedent sections that the electrochemical performance of the CNT-based electrodes was highly influenced by their whole preparation process which finally marked their own characteristics. In this section the electrostatic interactions and adsorptive effects of anionic hexacyanoferrate (III), cationic hexaammineruthenium (III) and neutral 1,4-benzoquinone with the different electrodes were explored by cyclic voltammetry experiments carried out from 10 to 255 mV s⁻¹. The voltammograms were analyzed and the relationships of peak currents and peak potentials with the scan rate were examined and they are shown in Tables 2–4.

Plots of peak current against the scan rate were linear only for hexacyanoferrate (III) at GC/(CNT/PEI) and hexaammineruthenium (III) at GC/(CNT/Nafion) as a result of the electrostatic interactions of analytes with the dispersing polymer. In the rest of the studied cases, peak currents were linear with the square root of scan rate indicating that the electrochemical processes were diffusion controlled. Cyclic voltammograms of inorganic complexes showed in all cases well-defined reduction and oxidation waves with slight increase in the peak potential difference when the scan rate is increased except for the above-mentioned cases where peaks separation largely increased with the scan rate probably due to the different electron transfer kinetics of the electrostatically adsorbed analytes. On the other hand, electrostatic repulsion resulted in higher peak potential differences for hexacyanoferrate (III) at GC/(CNT/Nafion) and hexaammineruthenium (III) at GC/(CNT/PEI) when compared to other electrodes which did not have electrostatic interactions with the analytes.

Table 3

Cyclic voltammetry of 1.0 mM hexaammineruthenium (III) in 0.1 M KCl

	i_p vs. $v^{1/2a}$	$\log i_p$ vs. $\log v^a$	ΔE_p vs. v^b
GC	$-0.7 - 1.44v^{1/2}$	$0.25 + 0.46\log v$	$68 + 0.040v$
GC/(CNT/PEI)	$7.6 - 7.1v^{1/2}$	$0.60 + 0.60\log v$	$58 + 0.19v$
GC/(CNT/EtOH)	$20 - 9.8v^{1/2}$	$1.40 + 0.67\log v$	$51 + 0.25v$
GC/(CNT/Nafion)	–	$1.16 + 0.94\log v$	$23 + 2.63v$
CPE	$0.4 + 1.87v^{1/2}$	$0.37 + 0.45\log v$	$62 + 0.003v$
CNTPE	$-0.3 - 2.19v^{1/2}$	$0.56 + 0.50\log v$	$61 + 0.014v$

^a i_p were in μA and v in mV s⁻¹.

^b ΔE_p were in mV and v in mV s⁻¹.

Table 4

Cyclic voltammetry of 0.80 mM 1,4-benzoquinone in 0.050 M phosphate of pH 7.40

	i_p vs. $v^{1/2a}$	$\log i_p$ vs. $\log v^a$	ΔE_p vs. v^b	αn_a^c
GC	$1.4 - 2.62v^{1/2}$	$0.51 + 0.46\log v$	$379 + 0.49v$	0.83
GC/(CNT/PEI)	$27 - 30.4v^{1/2}$	$1.43 + 0.51\log v$	$121 + 0.86v$	0.57
GC/(CNT/EtOH)	$26 - 31.4v^{1/2}$	$1.40 + 0.51\log v$	$151 + 0.90v$	0.48
GC/(CNT/Nafion)	$7.9 - 17.8v^{1/2}$	$1.16 + 0.54\log v$	$114 + 0.64v$	0.56
CPE	$-1.4 - 3.25v^{1/2}$	$0.58 + 0.47\log v$	$363 + 0.46v$	1.0
CNTPE	$0.9 - 3.46v^{1/2}$	$0.56 + 0.50\log v$	$374 + 0.39v$	1.0

^a i_p were in μA and v in mV s^{-1} .^b ΔE_p were in mV and v in mV s^{-1} . Calculated in the 50 to 255 mV s^{-1} interval.^c Calculated from 50 mV s^{-1} voltammograms.

A different situation was observed for 1,4-benzoquinone (see Fig. 2) where the reversibility of the reduction and reversal oxidation is highly compromised by the employed electrode. At bare GC and CPE the redox waves were separated about 400–500 mV, depending on the scan rate. At CNT-based electrodes, the peak separation notably decreased, being about 300 mV at CNTPE and from 100 to 300 mV depending on the scan rate at GC modified with CNT dispersions. This effect could be ascribed to the enhanced electron transfer rate of CNT. The observed reversibility is highly affected in all cases by the scan rate, increasing significantly the peak-to-peak separation at high

scan rates. It was also noticeable the difference between the peak shapes obtained at GC modified with CNT dispersions and those obtained at bare GC, CPE and CNTPE. In the former, peaks were tailed before reaching the top and decreased sharply after that while in the latter a typical diffusion tail was observed after the peak maximum. This behavior at the CNT layer is explained in the basis of their microscopic rough and porous structure, where the solution is partially occluded and the analyte replacement by diffusive transport during the electrochemical process is more difficult, thus the electrodes under these conditions (more pronounced at higher scan rates) had “thin-layer” characteristics [4]. On the opposite, the other electrodes did not have these transport impediments and typical diffusion profiles were observed. These effects were evident in the αn_a calculated from the logarithmic analysis of voltammograms [47] obtained at 50 mV s^{-1} where values closer to the electrochemical reversibility were found for GC, CPE and CNTPE electrodes while for GC modified with CNT dispersions these values were similar to electrochemically irreversible systems.

These experiments have demonstrated that the electron transfer kinetic of the studied electrochemical processes is faster at GC electrodes modified with CNT dispersions but their specific capacitances generate an overpotential associated to the double layer charge process that became more relevant as the scan rate is increased and enlarge rapidly the peak-to-peak separation.

3.4. Flow experiments in micellar media

The next step of this comparative study was the exploration of the amperometric response of the electrodes under flow conditions in the presence of SDS in concentration currently employed in micellar electrokinetic capillary chromatography (MEKC). The selected analytes for these experiments were hydrogen peroxide, amitrol, diuron and 2,3-dichlorophenol. Under these conditions all analytes were neutral and their different polarities determine their interaction with the micelles, in this way diuron and 2,3-dichlorophenol have a strong interaction, amitrol has little interaction and hydrogen peroxide has no interaction.

One of the most required qualities of electrodes for applying them in amperometric detection within separation systems is the long-term stability and, in this sense, electrodes with reproducible responses and antifouling properties are desirable. Thus, the stability of the amperometric response of the electrodes was studied by following the signal evolution during 35 min of con-

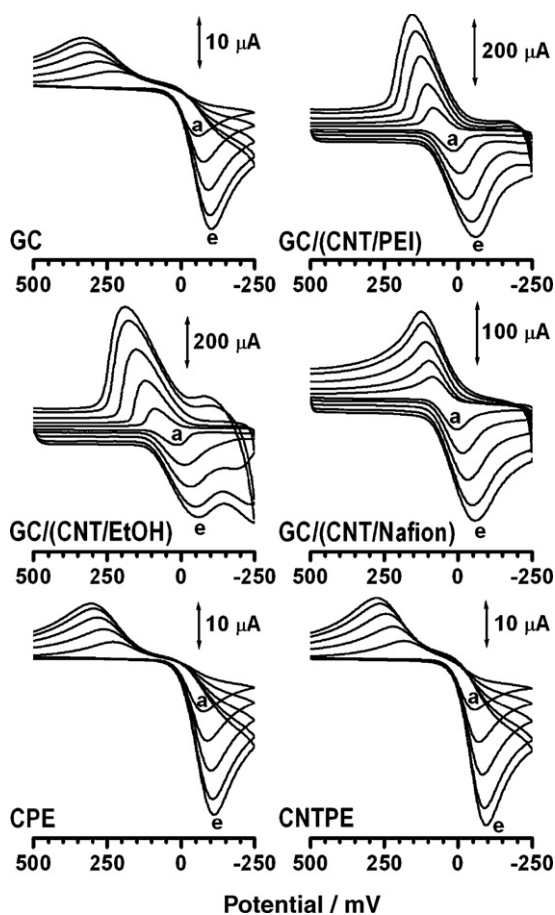


Fig. 2. Cyclic voltammograms of 0.80 mM 1,4-benzoquinone in 0.050 M phosphate buffer of pH 7.40 at 10 (a), 25 (b), 50 (c), 80 (d) and 100 mV s^{-1} (e). Solutions were purged with N_2 for 15 min.

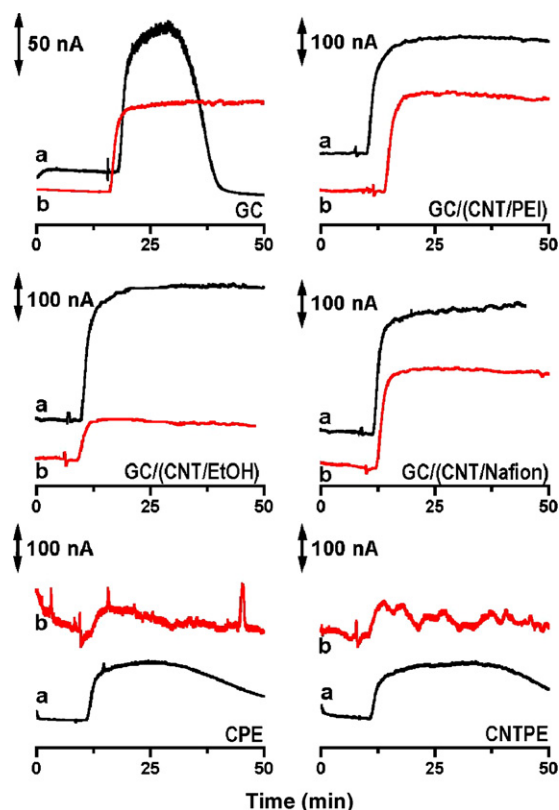


Fig. 3. Stability of the amperometric signal to continuous exposure of $15 \mu\text{M}$ 2,3-dichlorophenol at 0.5 mL min^{-1} . Carrier electrolyte, 0.050 M phosphate buffer of pH 7.40, black line (a) and 0.050 M phosphate buffer of pH 7.40 with 20 mM SDS, red line (b). Detection potential, $+800 \text{ mV}$. In order to facilitate the comparison of signals at CPE and CNTPE, those obtained in the presence SDS were offset $1 \mu\text{A}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

tinued exposure to the model analytes at 0.5 mL min^{-1} flow rate in 0.050 M phosphate buffer of pH 7.40 in the presence and in the absence of 20 mM SDS. The amperometric recordings of 2,3-dichlorophenol are shown in Fig. 3 as a representative example of these experiments. From a general view it was noticed that background currents at paste electrodes were strongly affected by the presence of SDS, increasing largely (from tens of nA to μA ; note the offset of SDS signals at CPE and CNTPE) and longer stabilization times were needed as a result of the surfactant incorporation to the mineral oil binder that increased the capacitance of these electrodes. On the other hand SDS had negligible effect upon the background of bare GC and GC modified with CNT dispersions so flat baselines were obtained at a reasonable time.

For hydrogen peroxide and amitrol, which have weak interaction with the micellar media, stable signals were obtained at $+700 \text{ mV}$ in the presence and in the absence of SDS while no fouling symptom was noticed in all cases. The presence of SDS increased the amperometric signals at paste electrodes while they decreased at bare and GC modified with CNT dispersions. In these cases, the kinetic of the electrochemical processes was enhanced by the massive incorporation of SDS to paste electrodes surface while the different surfactant interaction at GC and GC modified electrodes had the opposite effect.

Diuron showed stable oxidation signals at $+900 \text{ mV}$ in all cases in the absence of SDS but, under these conditions, no defined signals were distinguished from the high background at paste electrodes in the presence of surfactant. At bare GC and GC modified with CNT dispersions, the presence of SDS had adverse effect once again upon the amperometric signals. Diuron had a strong interaction with the micelles and the electron transfer could be more difficult when the pesticide is inside their hydrophobic core.

The amperometric signals for 2,3-dichlorophenol oxidation at $+800 \text{ mV}$ in the absence of SDS showed the surface passivation of bare GC, CPE and CNTPE electrodes while at GC modified with CNT dispersions stable responses were obtained. The presence of surfactant resulted in a decrease of the passivation occurrence accompanied by a diminution of GC, CPE and CNTPE signals. Measurements at paste electrodes were also difficult due to the dramatic background increase. The signals diminution at GC modified with CNT dispersions was lighter and no surface fouling was observed.

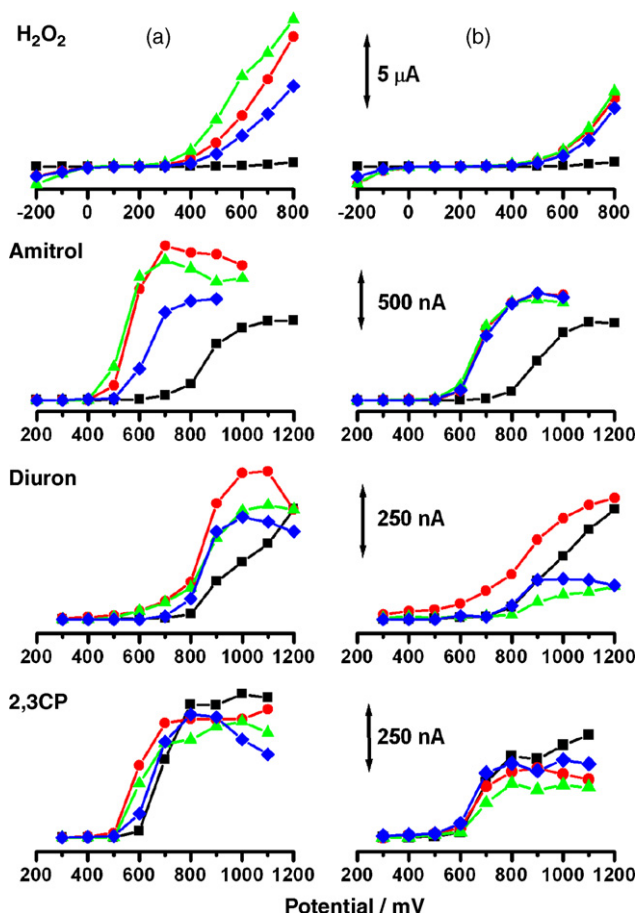


Fig. 4. Hydrodynamic voltammograms of 2.0 mM hydrogen peroxide, $15 \mu\text{M}$ amitrol, $15 \mu\text{M}$ diuron and $15 \mu\text{M}$ 2,3-dichlorophenol (2,3CP) at GC (black squares), GC/(CNT/PEI) (red circles), GC/(CNT/EtOH) (green triangles) and GC/(CNT/Nafion) (blue diamonds). Flow rate, 2.0 mL min^{-1} . Carrier electrolyte, 0.050 M phosphate buffer with pH 7.40 (column a) and 0.050 M phosphate buffer with pH 7.40 and 20 mM SDS (column b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

The results obtained in this study have demonstrated the superior electrochemical performance of GC modified with CNT dispersions under flow regime in the presence SDS, showing good stability and antifouling properties. On the opposite, paste electrodes were discarded for the next studies as a result of the adverse effects of SDS upon their background signals and the observed faster passivation.

In order to obtain more insights about the electrochemical performance of GC electrodes modified with CNT dispersions, the hydrodynamic voltammograms of the model analytes (100 μL loop samples) were constructed at 2.0 mL min^{-1} flow rate, in the presence and in the absence of 20 mM SDS and the results obtained are displayed at Fig. 4.

For hydrogen peroxide and amitrol, the presence of the CNT layer supposed an enhancement upon their oxidation processes when compared to bare GC in the absence of SDS, with potential shifts of 300 mV towards lower potentials, observed at the feet of the waves, and lower sensibilities at GC/(CNT/Nafion). The hydrogen peroxide electrochemical reduction was also improved in a similar way at CNT-based electrodes. In the presence of SDS, a positive potential shift of about 100 mV was noticed for hydrogen peroxide and amitrol oxidation at all the tested electrodes, suggesting that the electron transfer processes were more difficult under these conditions. Moreover, the sensitivities of CNT-based electrodes decreased and became similar. The hydrogen peroxide reduction process was not significantly affected by the surfactant.

Diuron hydrodynamic voltammograms obtained at CNT-based electrodes in the absence of SDS showed oxidation

signals starting at 600 mV (GC/(CNT/PEI)), 700 mV (GC/(CNT/EtOH)) and 800 mV (GC/(CNT/Nafion)) which reached a plateau at 1000 mV. At bare GC diuron oxidation started at 800 mV and the plateau was not attained under these conditions, with a sensitivity lower than those observed at GC/(CNT/PEI) and GC/(CNT/EtOH). In this case, the diuron oxidation at CNT is favored but the sensitivity was affected by the charge of the dispersing polymer. When SDS was added to the carrier electrolyte, the sensitivities of all electrodes were lowered, specially at GC/(CNT/Nafion) and GC/(CNT/EtOH), while the shape of GC/(CNT/PEI) was slightly tailed. Under these conditions GC/(CNT/PEI) had better performance than the other dispersions and the bare GC.

Finally, the voltammograms of 2,3-dichlorophenol obtained without SDS showed that the pollutant oxidation began at CNT-based electrodes at about 500 mV, increasing rapidly at GC/(CNT/PEI) and GC/(CNT/EtOH) and reaching a plateau at 700 mV. This increase was lighter at GC/(CNT/Nafion), reaching the plateau at 800 mV. Oxidation at GC started at 600 mV and the plateau was reached at 800 mV. In the presence of SDS, the sensitivity of the amperometric signals fell to the half and the voltammograms were similar at all the tested electrodes, starting at 600 mV and reaching the plateau at 800 mV.

In the light of the results obtained, GC/(CNT/PEI) has proven to be the most suitable CNT-based electrode for its application as amperometric detector for MEKC separations as a result of its enhanced electrochemical performance and antifouling properties which are maintained in the presence of SDS.

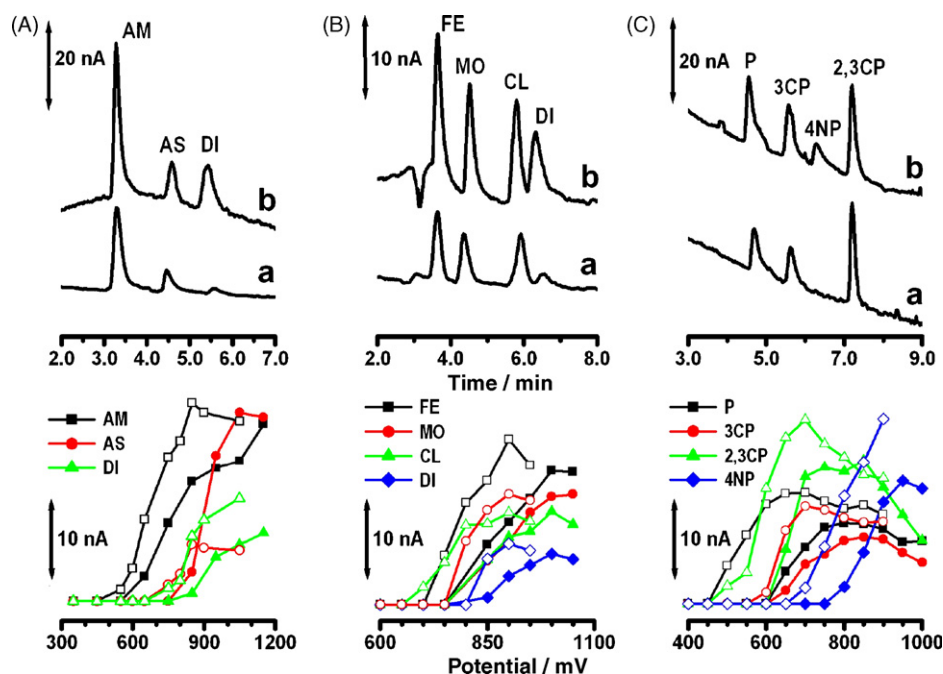


Fig. 5. MEKC separations of herbicides (A), phenylurea herbicides (B) and phenolic pollutants (C) at GC (a, bold symbols) and GC/(CNT/PEI) (b, hollow symbols) along with the corresponding hydrodynamic voltammograms. Separation buffer, 10 mM phosphate buffer of pH 7.40 with 20 mM SDS. Separation voltage, 20.0 kV. Hydrodynamic sample introduction for 1.5 s (79 nL). Sample concentrations: 50 μM Amitrol (AM), Asulam (AS) and Diuron (DI) in (A); 50 μM Fenuron (FE), Monuron (MO), Chlortoluron (CL) and Diuron (DI) in (B); 100 μM phenol (P) and 3-chlorophenol (3CP), 200 μM 4-nitrophenol (4NP) and 2,3-dichlorophenol (2,3CP). Detection potentials, +850 mV (A and B) and +750 mV (C).

3.5. MEKC separations of pollutants

The efficient performance in micellar media of GC electrodes modified with CNT dispersions has been demonstrated in the precedent sections, showing their promising application as amperometric detector in micellar electrokinetic capillary chromatography (MEKC) separations. In order to illustrate this potential, three different MEKC separations of the herbicides amitrol, asulam and diuron, the phenolic pollutants phenol, 2-chlorophenol, 2,3-dichlorophenol and 4-nitrophenol and the phenylurea herbicides fenuron, monuron, chlortoluron and diuron were carried out in 10 mM phosphate buffer of pH 7.40 containing 20 mM SDS. The amperometric detection of these separations was done using GC/(CNT/PEI) and bare GC electrodes and their responses under these conditions were compared. Fig. 5 displays typical electropherograms obtained at bare GC (a) and GC/(CNT/PEI) (b) along with the corresponding hydrodynamic voltammograms. Separations of the tested pollutants were attained with good resolution and well-defined peaks were observed in all cases. It could be observed the shift to lower potentials (100–150 mV in most of the cases) in the hydrodynamic voltammograms at GC/(CNT/PEI) when compared to those obtained at bare GC. The facilitated electrochemical processes at CNT-based electrode resulted in higher sensitivities at the selected detection potentials while a reasonable background signal was maintained. For example diuron and 4-nitrophenol were hardly detected at bare GC at these potentials while well-defined peaks were obtained at GC/(CNT/PEI) in the same conditions. In addition, the sensitivities of amitrol, fenuron monolinuron and chlortoluron were the double at GC/(CNT/PEI) than those obtained at bare GC. In this way the limits of detection calculated under these conditions using the $S/N = 3$ criterion were 3 μM for amitrol, 7 μM for fenuron and monuron and 9 μM for chlortoluron respectively at GC/(CNT/PEI) while 10 μM for amitrol and 20 μM for fenuron, monuron and chlortoluron, respectively, were estimated at GC. Other important feature was the observed stability of the GC/(CNT/PEI) signals which was specially remarkable for the phenolic pollutants detection since it is well known the important surface electrode poisoning suffered when these compounds are oxidized (RSD after 10 consecutive sample introductions were below 3.1% and 8.7% for migration times and peak heights, respectively). The advantages provided by the use of the CNT-based electrode are evident since their electrocatalytic and antifouling properties are maintained in micellar media expanding the detection possibilities in MEKC separations.

4. Conclusions

This comparative study has pointed the different electrochemical activity of CNT-based electrodes depending on the methodology employed for their preparation. Surface morphology, active surface area and specific capacitance differ significantly from paste electrodes to GC electrodes modified with CNT dispersions, reflecting the different distribution and availability of active sites within these electrodes that finally

defines their electrocatalytic activity. The presence of SDS in the electrolyte has been demonstrated having a dramatic influence upon the paste electrodes background signal that difficult the practical use of these electrode under these conditions. This adverse effect is not observed at GC modified electrodes while their electrochemical activity remains almost unaltered in the presence of the surfactant. The CNT-dispersing reagents influence the electrochemical properties of the resulting electrodes, where electrostatic interactions have a relevant role when charged and neutral analytes were analyzed as well as the individual electrochemical mechanism. Among the tested electrodes, GC electrodes modified with CNT dispersions in PEI have displayed enhanced electrochemical performance after all the experiments carried out and they were then applied for the amperometric detection in MEKC separations. These CNT-based electrodes have demonstrated displaying the same superior electrochemical performance and antifouling properties in this microfluidic environment and their utility is presented for the proposed separations of herbicides and phenolic pollutants.

Congratulation

We would like to use this opportunity to congratulate Dr. Joseph Wang on the occasion of his 60th birthday. Happy Birthday Dr. Wang. The time that we spent with him in his laboratory (M. Chicharro in the Lab. Sensor of New Mexico State University (1995–1996), and A. Sánchez in the Center for Bioelectronics and Biosensors within the Biodesign Institute of Arizona State University (2004–2005)) was an unique especial opportunity for us. Thank you very much for everything.



M. Chicharro

A. Sánchez

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References

- [1] S. Iijima, Nature 354 (1991) 56–58.
- [2] P.M. Ajayan, Chem. Rev. 99 (1999) 1787–1800.
- [3] H. Dai, Surf. Sci. 500 (2002) 218–241.
- [4] C.E. Banks, R.R. Moore, T.J. Davies, R.G. Compton, Chem. Commun. 16 (2004) 1804–1805.
- [5] N.S. Lawrence, R.P. Deo, J. Wang, Electroanalysis 17 (2005) 65–72.
- [6] C.E. Banks, T.J. Davies, G.G. Wildgoose, R.G. Compton, Chem. Commun. 17 (2005) 829–841.

- [7] M. Musameh, J. Wang, A. Merkoci, Y. Lin, *Electrochem. Commun.* 4 (2002) 743–746.
- [8] K. Jurkschat, X. Ji, A. Crossley, R.G. Compton, C.E. Banks, *Analyst* 132 (2007) 21–23.
- [9] M. Pumera, *Langmuir* 23 (2007) 6453–6458.
- [10] N.S. Lawrence, R.P. Deo, J. Wang, *Talanta* 63 (2004) 443–449.
- [11] G.G. Wildgoose, C.E. Banks, H.C. Leventis, R.G. Compton, *Microchim. Acta* 152 (2006) 187–214.
- [12] B. Sljukic, C.E. Banks, R.G. Compton, *Nano Lett.* 6 (2006) 1556–1558.
- [13] C.E. Banks, A. Crossley, C. Salter, S.J. Wilkins, R.G. Compton, *Angew. Chem. Int. Ed.* 45 (2006) 2533–2537.
- [14] X. Dai, G.G. Wildgoose, R.G. Compton, *Analyst* 131 (2006) 901–906.
- [15] M.D. Rubianes, G.A. Rivas, *Electrochem. Commun.* 5 (2003) 689–694.
- [16] F. Valentini, A. Amine, S. Orlanduci, M.L. Terranova, G. Palleschi, *Anal. Chem.* 75 (2003) 5413–5421.
- [17] J. Wang, M. Musameh, *Anal. Chem.* 75 (2003) 2075–2079.
- [18] M. Pumera, A. Merkoçi, S. Alegret, *Sens. Actuators B* 113 (2006) 617–622.
- [19] J.J. Gooding, *Electrochim. Acta* 50 (2005) 3049–3060.
- [20] A. Merkoçi, M. Pumera, X. Llopis, B. Pérez, M. del Valle, S. Alegret, *Trends Anal. Chem.* 24 (2005) 826–838.
- [21] J. Wang, M. Musameh, Y. Lin, *J. Am. Chem. Soc.* 125 (2003) 2408–2409.
- [22] G.A. Rivas, S.A. Miscoria, J. Desbrieres, G.D. Barrera, *Talanta* 71 (2007) 270–275.
- [23] M. Zhang, A. Smith, W. Gorski, *Anal. Chem.* 76 (2004) 5045–5050.
- [24] S. Bollo, N.F. Ferreyra, G.A. Rivas, *Electroanalysis* 19 (2007) 833–840.
- [25] Ch. Wang, Z.-X. Guo, S. Fu, W. Wu, D. Zhu, *Prog. Polym. Sci.* 29 (2004) 1079–1141, and references therein.
- [26] M.D. Rubianes, G.A. Rivas, *Electrochem. Commun.* 9 (2007) 480–484.
- [27] J. Wang, *Electroanalysis* 17 (2005) 1133–1140.
- [28] J. Wang, G. Chen, M.P. Chatrathi, M. Musameh, *Anal. Chem.* 76 (2004) 298–302.
- [29] M. Chicharro, A. Sánchez, E. Bermejo, A. Zapardiel, M.D. Rubianes, G.A. Rivas, *Anal. Chim. Acta* 543 (2005) 84–91.
- [30] M. Vlčková, M.A. Swartz, *J. Chromatogr. A* 1142 (2007) 214–221.
- [31] M. Pumera, A. Merkoçi, S. Alegret, *Electrophoresis* 28 (2007) 1274–1280.
- [32] J. Wang, G. Chen, M. Wang, M.P. Chatrathi, *Analyst* 129 (2004) 512–515.
- [33] A. Sánchez Arribas, E. Bermejo, M. Chicharro, A. Zapardiel, G.L. Luque, N.F. Ferreyra, G.A. Rivas, *Anal. Chim. Acta* 577 (2006) 183–189.
- [34] G. Chen, L.Y. Zhang, J. Wang, *Talanta* 64 (2004) 1018–1023.
- [35] X. Yao, X. Xu, P. Yang, G. Chen, *Electrophoresis* 27 (2006) 3233–3242.
- [36] M. Pumera, X. Llopis, A. Merkoçi, S. Alegret, *Microchim. Acta* 152 (2006) 261–265.
- [37] X. Yao, H. Wu, J. Wang, S. Qu, G. Chen, *Chem. Eur. J.* 13 (2007) 846–853.
- [38] C.W. Huck, G. Stecher, H. Scherz, G. Bonn, *Electrophoresis* 26 (2005) 1319–1333.
- [39] B. Baena, A. Cifuentes, C. Barbas, *Electrophoresis* 26 (2005) 2622–2636.
- [40] D. Fliser, S. Wittke, H. Mischak, *Electrophoresis* 26 (2005) 2708–2716.
- [41] A.E. Kaifer, A.J. Bard, *J. Phys. Chem.* 89 (1985) 4876.
- [42] X.L. Wen, Y.-H. Jia, Z.-L. Liu, *Talanta* 50 (1999) 1027–1033.
- [43] A.J. Bard, L.R. Faulkner, *Electrochemical Methods. Fundamentals and Applications*, second ed., John Wiley & Sons, New York, 2000, Chapter 1.
- [44] M. Chicharro, E. Bermejo, M. Moreno, A. Sánchez, A. Zapardiel, G. Rivas, *Electroanalysis* 17 (2005) 476–482.
- [45] J. Zhang, S. Yao, L. Nie, W. Wei, *Anal. Sci.* 16 (2000) 87–91.
- [46] M. Pumera, *Nanoscale Res. Lett.* 2 (2007) 87–93.
- [47] C.E. Banks, R.G. Compton, *Analyst* 130 (2005) 1232–1239.

Review

Real sample analysis on *microfluidic* devices[☆]Agustin González Crevillén, Miriam Hervás, Miguel Angel López,
Maria Cristina González, Alberto Escarpa^{*}Department of Analytical Chemistry and Chemical Engineering, University of Alcalá,
E-28871 Alcalá de Henares, Madrid, Spain

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Abstract

This review covers the state of the art of the analysis of real (or non-ideal) samples on *microfluidic* devices. A real sample analysis performed on *microfluidics* conceptually involves the complete integration of sample preparation, analyte separation, and detection on these platforms. Different “lab-on-a-chip” approaches have emerged in relevant application areas such as clinical, environmental, and food analysis which will be critically illustrated and discussed with respect to the strengths and weakness found. Likewise, the main challenges and perspectives will also be commented on.

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Keywords: *Microfluidics*; Lab-on-a-chip; Real sample analysis; Clinical; Environmental; Food samples

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1. Introduction

The micro total analysis system (μ -TAS) concept, later called “lab-on-a-chip,” was developed from the modification of the total analysis system (TAS) approach by downsizing and integrating its multiple steps (injection, reaction, separation, and detection) onto a single device, yielding a sensor-like system with a fast response time, low sample consumption, on-site operation, and high stability [1].

Miniaturization is more than simply the scaling down of well-known systems since the relative importance of forces and processes changes with scale. One of the most relevant characteristics of analytical microsystems is the omnipresence of laminar flow (Reynold’s number are typically very low), in which viscous forces dominate over inertia. This means that turbulence is often unattainable and that molecule transportation only occurs through diffusion, which has direct consequences on the designs of this type of microsystem. The other relevant feature is the possibility of handling fluidics on the nanoliter and even picoliter scale, which has widened the scope of micro-TAS to now be called *microfluidics*. *Microfluidics* is the science and technology of systems that process or manipulate small amounts of fluidics (10^{-9} to 10^{-18} L), using channels measuring from tens to hundreds of micrometers [2,3]. For this reason,

[☆] Published in Honor of Professor Joseph Wang’s 60th Birthday.^{*} Corresponding author.E-mail address: alberto.escarpa@uah.es (A. Escarpa).

the term *microfluidics* better covers the research and emphasizes the strong impact miniaturization and integration have on the fluidics and chemical engineering of analytical microsystems.

Microfluidics in analytical chemistry can be seen as different analytical microsystems developed according to the analytical objectives clearly based on sample complexity and selectivity criteria [4]: (i) the direct measurement of one or a few components with little or no sample preparation ((bio) sensors, probe-type sensors, flow-through sensors, and microsensors); (ii) the measurement of one or a few components which require some treatment of the sample (μ FIA); (iii) the analysis of more complex samples involving the separation of their components (μ -HPLC and μ -CE).

Parallely, and in connection with this development, it is important to point out that not all analytical steps and/or microsystems are equally incorporated and the incidence level of miniaturization is not homogeneous. In fact, the detection and separation steps are the ones most investigated, whereas sample preparation is notably the least developed [5]. From the beginning, detection has been one of the main challenges for analytical microsystems, since very sensitive techniques are needed as a consequence of the ultra-small sample volumes used in micron-sized environments. In principle, a wide variety of detection alternatives can be used in *microfluidic* systems, but laser-induced fluorescence (LIF) and electrochemical detection (ED), primarily, along with mass spectrometry (MS) are the routes most commonly used. LIF was the original detection technique and it is the most often used detection scheme in CE microchips because of its inherent sensitivity [6]. However, the high cost and the large size of the instrumental setup for LIF are sometimes incompatible with the concept of μ TAS. In addition, tedious derivatization schemes are needed to use LIF with non-fluorescent compounds. The principal alternative to LIF detection is, without a doubt, ED. ED is very important because of its inherent miniaturization without loss of performance and its high compatibility with microfabrication techniques. Likewise, it possesses high sensitivity, its responses are not dependent on the optical path length or sample turbidity, and it has low power supply requirements which are additional advantages. Proof of the predominant role of ED is the publication of several reviews [7–9]. Apart from LIF and ED, there are other detection approaches for analytical microsystems, but from a realistic point of view, they have been developed to a lesser extent. These unconventional detection routes have recently been revised and include, among others, infrared, Raman, NMR, surface plasmon resonance (SPR), absorbance, thermal lens, and chemiluminescence detectors [10].

With respect to separation techniques, micro-CE was one of the earliest examples of μ TAS, and it constitutes one of the most representative examples of analytical microsystems [11]. Using CE microchips, analysis times can be reduced to seconds and extremely high separation efficiencies can be achieved. The easy microfabrication of a network of channels using materials of well-known chemistry, which themselves have good electroosmotic flows, and the possibility of using the electrokinetic phenomena to move fluids are among the most important

factors to understand the relevance of CE microchips to miniaturization. Since electrokinetics is easy to apply (only a pair of electrodes are needed), electroosmotic-driven flow (EOF) has been successfully implemented using different types of materials to manufacture the channels, being glass the most commonly used. Microfabrication on polymers is faster and cheaper than on glass, so these materials have great potential for mass production. However, glass chips present the best EOF and the chemical modification on the surfaces of its microchannels is better known and easier compared to polymers. Many of the benefits already discussed for CE microchips could equally be applied to downsized chromatographic techniques; however, relatively few examples of chip-based chromatographic instruments are covered in the literature compared with chip-based CE devices. This fact is not surprising since CE is almost perfectly suited to miniaturization. Also, the miniaturization of chromatographic systems involves a number of technical challenges, such as the microfabrication of valves and pumps, which are generally not encountered in CE [5].

Sample preparation steps involving emerging technology for sample preparation/pre-treatment [12] and the macroworld to microworld interfaces [13,14] have been addressed. Because of its inherent complexity, the sample preparation step is less developed than those of separation and detection. However, a very exciting future in real analysis is conceivable because of (i) the inherent possibilities of microfabrication technology to create sophisticated designs (complex layouts according to sample requirements) and microstructures (filters) in connection with sample preparation requirements; (ii) the inherent possibilities offered by *microfluidics*: the presence of laminar flow and diffusion in action (H-filter example [15]); and (iii) the ease of using electrokinetic phenomena to move fluidics into the network channels with accuracy (focusing on flow techniques).

In addition, other important general challenges still remain awaiting analytical solutions such as automation, sampling, sample introduction, and sample preparation (except derivatization schemes). Moreover, a breakthrough in the so-called *real world interface* is highly anticipated [5]. Along these lines, total integration and, especially, world-to-chip interfacing are considered the major challenges, particularly in high-throughput applications requiring frequent sample changes, such as continuous *on-line* process monitoring.

From an analytical point of view, the analysis of real samples is often the ultimate goal of a target study. Although the volume of research in this area is impressive, real sample analysis is still in its infancy and constitutes one of the major challenges of *microfluidics*. As a consequence, analyses of true real samples using analytical microsystems, *microfluidics*, or the microchip format are very scarce. This review provides in-depth coverage of the *state of the art* of *microfluidic* devices, focusing on the analysis of real samples as indicators of the grade of maturity of these microsystems. In this context, although every sample is a “real sample,” by the analysis of a real sample we mean the analysis performed directly on the matrix containing the analyte (or those spiked). In addition, we understand *microfluidic* devices to be those miniaturized systems for (total) (bio) chemical analysis. These systems are interconnected networks of channels and

reservoirs containing nano- and microliter volumes. An important subset/subclass of these *microfluidic* devices, namely CE microchips, are those made on glass or polymers using electrokinetic techniques for fluidic motivation and analyte separation. Given the large number of publications in the area, this review is constructed around clinical, environmental, and food analysis while other areas, such as molecular analysis (DNA and RNA) [16], forensics [17], and biodefense [18,19], are not covered in detail and readers are encouraged to read these excellent reviews. In sum, the main challenges found and the proposed approaches for clinical, environmental, and foods areas in the sample analysis domain using *microfluidic* devices will be critically discussed.

2. Real clinical sample analysis on *microfluidics*

Clinical and biochemical analysis have been the most extensive fields of application for lab-on-a-chip systems and currently they continue to grow [20–23]. The measurement of relevant analytes in physiological fluids for the diagnosis and prevention of diseases is a very exciting application, especially in point-of-care testing. The advantages (cited above) of downsizing from TAS to miniaturized devices are ideal in the case of near-patient clinical analysis. Smaller patient sample volume, which is particularly important in geriatric and pediatric patients; reduced reagent consumption; shortened analysis time; integrated multiple processes in a single device (portability); and enhanced reliability and sensitivity through automation are just some of the achievable benefits which make this technology an interesting approach.

Nevertheless, as was already mentioned, not all of the processes of an analytical procedure are equally incorporated into a miniaturized device (lab-on-a-chip). In clinical analysis, many reports have been published on the detection step in *microfluidic* systems, whereas the sample preparation step tends to be accomplished *off-chip* [24,25]. Often, the analyte of interest is accommodated within an extremely complex matrix (for example blood) which requires different sample pre-treatment techniques, making it difficult to incorporate into a microchip, this being the main obstacle in processing real samples, and to achieve a true micro-TAS device.

Working with real samples usually requires some degree of purification (isolation/clean-up) and preconcentration prior the measurement step. Sensitivity (only a few nanoliters reach the detector) and selectivity can pose a serious problem in the analysis of these raw samples. However, in the field of clinical miniaturized analysis, the use of enzymes, antibodies, and other biological reagents saves extensive sample pre-treatment. The sensitivity associated to the use of enzymes allows samples to be diluted to eliminate interference from other constituents of the matrix or adsorption problems of proteins to the capillary walls without lowering detection limits. Conversely, the use of antibodies, enzymes, or other affinity receptors are responsible for the selectivity required for these assays.

A wide variety of bioanalytical and clinically relevant analytes, such as peptides, amino acids, neurotransmitters, carbohydrates, and vitamins, have been determined in the microchip

format. However, references describing analysis in complex matrices are few, most of them being laboratory prepared samples.

This section of the review focuses on those analytes determined in true real or spiked samples. Selected examples will be described from different approaches such as the use of capillary electrophoresis, enzymes, and antibodies to provide selectivity to the assay. The most remarkable features and the type of pre-treatment accomplished are outlined.

Table 1 lists the most relevant information about the *microfluidic* approaches used in the analysis of clinical samples.

The majority of the existing miniaturized systems require highly predictable and homogenous samples. In the case of heterogeneous samples, such as human whole blood, cell-free samples such as serum or plasma are preferable over those with problems associated with flow-path blockage and cell lysis. In this case, whole blood filtration is accomplished *on-chip* for the subsequent analysis. Thorslund et al. [26] have developed a microsystem for *on-chip* sample preparation with which testosterone is measured in diluted whole blood. This system uses a hydrophilic polypropylene membrane filter incorporated onto a PDMS device to remove blood cells with minimal adsorption of proteins.

In clinical analysis, microchip capillary electrophoresis is the most common technique used in miniaturized analytical devices due to its special suitability for miniaturization [27–29]. Relevant analytes used to diagnose different diseases have been analyzed by microchip capillary electrophoresis. Lipoprotein analysis in serum samples was demonstrated by Verpoorte's group [30,31]. In their first approach [30], low density (LDL) and high density (HDL) lipoproteins were separated in a glass microchannel using a dynamic coating (methylglucamine) to prevent lipoprotein adsorption. NBD-ceramide was used as a label for laser induced fluorescence detection. In a later study [31], they demonstrate the analysis of different forms of clinically relevant LDL lipoproteins without the need for a permanent or dynamic coating of the glass microchannels. Adding SDS below the critical micelle concentration to the sample provided sufficient sample recovery to detect the lipoprotein at low concentrations. In both cases, ultracentrifugation of the serum and its subsequent dialysis was accomplished prior microchip analysis.

Neurotransmitters and their metabolites have been determined by microchip CE in biological fluids. Simultaneous amperometric and fluorescence detection is reported by Lapos et al. for the analysis of cerebrospinal fluid samples (CSF) [32]. The use of an orthogonal detection scheme allows LIF detection of NBD-arginine and NBD-phenylalanine, and electrochemical detection of dopamine and catechol, increasing the possibilities for the detection of analytes with different physical characteristics. The use of two detectors simultaneously reduces the run-to-run migration time irreproducibility for certain samples. CSF samples from patients with multiple sclerosis were centrifuged, diluted, and spiked with electroactive species *off-chip*. Johirul et al. [33] have developed a *microfluidic* chip based on CE coupled with a cellulose-ssDNA modified screen-printed electrode for the simultaneous analysis of dopamine (DA), norepinephrine (NE),

Table 1
Microfluidic approaches used in real clinical analysis

Sample	Analytes	Sample preparation	Microfluidic approach	Analytical characteristics	Remarks	Ref.
Whole blood (spiked)	Testosterona	Dilution <i>off-chip</i>	PDMS chip with membrane filter incorporated, RIA detection <i>off-chip</i>	Sample preparation microsystem	Sample preparation <i>On-chip</i> . Removing of blood cells with minimal adsorption of proteins.	[26]
Serum	Lipoproteins (LDL and HDL)	Ultracentrifugation and dialysis <i>off-chip</i>	Glass CE microchip, LIF detection	Separation time: 28 s	Methylglucamine as dynamic coating	[30]
Serum	Lipoproteins (LDL types)	Ultracentrifugation and dialysis <i>off-chip</i>	Glass CE microchip, LIF detection	Separation time: 21 s	Uncoated glass microchip	[31]
Cerebral spinal fluid (spiked)	Dopamine, catechol, NBD-arginine and NBD-phenylalanine	Centrifugation and dilution <i>off-chip</i>	Glass CE microchip, LIF and EC detection	LOD dopamine: 448 nM, LOD catechol: 1.52 μ M, LOD NBD-argin.: 16 μ M, LOD NBD-phenyl: 28 μ M	Dual fluorescence and electrochemical detection	[32]
Serum	Neurotransmitters (dopamine and ascorbic acid)	No treatment	Glass CE microchip, electrochemical detection	Separation time: 220 s, 1.3 (%RSD), LOD: 0.032 μ M	Cellulose-ssDNA modified SP electrode	[33]
Mouse brain and human urine	Catecholamines and their cationic metabolites	Dilution and filtration <i>off-chip</i>	Glass CE microchip, electrochemical detection	Separation time: 150 s, LOD: sub μ M	Simultaneous determination. Carbon nanotubes (CNT) modified electrodes	[34]
Urine	Amino acids	Filtration and dilution <i>off-chip</i>	Glass CE microchip, LIF detection	Separation time: 120 s, LOD: 32.9 μ M	Indirect fluorescence detection approach, 19 amino acids detected	[35]
Urine	Uric acid	Filtration and dilution <i>off-chip</i>	PDMS/Glass CE microchip, electrochemical detection	Separation time: 30 s, LOD: 1 μ M	50–75-Fold dilution avoid potential interferences	[36]
Urine	Creatine, creatinine, <i>p</i> -aminohippuric acid and uric acid	Dilution and filtration <i>off-chip</i>	Glass CE microchip, electrochemical detection	Separation time: 340 s, LOD creatine: 20 μ M, LOD uric acid: 40 μ M, LOD <i>p</i> -aminohippuric acid: 20 μ M	Coupling of enzymatic bioassay and electrophoretic separation	[37]
Urine (spiked)	Creatine, creatinine and uric acid	Dilution <i>off-chip</i>	PDMS CE microchip, electrochemical detection	Separation time: 200 s, LOD creatine: 250 μ M, LOD creatinine: 80 μ M, LOD uric acid: 270 μ M	Direct PAD detection	[38]
Serum and blood	Lactate and glucose	Dilution and filtration <i>off-chip</i>	Glass CE microchip, post-column enzymatic reaction, electrochemical detection	LOD glucose: 30 μ M, LOD lactate: 45 μ M	100-Fold dilution avoid protein adsorption	[39]
Plasma	Glucose	Dilution and filtration <i>off-chip</i>	PDMS/glass CE microchip, electrochemical detection	Separation time: 120 s, LOD: 6 μ M	–	[40]
Serum, plasma, urine and saliva (spiked)	Glucose	Dilution	Glass microchip, colorimetric enzymatic method	–	High degree of integration, electrowetting for microdroplet actuation	[41]
Serum (spiked)	Lactate	Dissolved	Integrated flow cell on silicon chip, chemiluminiscent enzymatic FIA system	Analysis time: 30 s, LOD: 0.5 mM	High degree of integration	[42]
Serum (spiked)	Ca ²⁺	No treatment	Glass/PDMS microchip, reflectance measurements	Analysis time: 30 s, LOD: 26.8 μ M	No significant interferences from other ions presented	[44]
Blood (spiked)	Hemoglobin	Dilution	Silicon microchip, photometric detection	Concentration range: 12–17 g/dL	Optical path extension with 45° mirrors at each end of the channel	[45]
Serum (spiked)	Tetanus antibody and tetanus toxin	No treatment	Glass microchip. PAGE gel CE immunoassay, LIF detection	Separation time: 3 min, LOD: 680 pM	Non-competitive and competitive immunoassay format, <i>off-chip</i> incubation	[47]
Saliva (spiked)	MMP-8	Centrifuged and diluted	Glass microchip. PAGE gel CE immunoassay, LIF detection	Analysis time: <10 min, LOD: 130 ng/mL	Pretreatment on chip (filtering, enrichment and mixing)	[48]
Tissue fluids	Neuropeptides	Ultramicrodialyzer, immunoextraction <i>on-chip</i>	Glass microchip, immunoaffinity CE, LIF detection	Separation time: 160 s, LOD: 0.6 pg/mL	12 neuropeptides are determined simultaneously	[49,50]
Live cells	Insulin	Conditioning and stimulation <i>On-chip</i>	Glass CE microchip, immunoassay, LIF detection	Assay time: 30 s, LOD: 3 nM	Competitive immunoassay format	[51]
Whole blood	Histamine	No treatment	PMMA microchip, electrochemical detection	Analysis time: 2 min, LOD: 200 ng/mL	Multichanneled matrix column coated with cation-exchange resin	[52]
Serum (spiked)	Carcinoembryonic antigen	No treatment	PMMA microchip, immunosorbent assay, thermal lens detection	Analysis time: 35 min, LOD: 0.03 ng/mL	Sandwich format on glass beads, antibody-colloidal gold conjugate	[53]
Serum (spiked)	Immunosuppressive acidic protein	Dilution	Quartz microchip, immunosorbent assay, chemiluminescence detection	Analysis time: 2 min, LOD: 0.1 μ M	Competitive format on glass beads, high degree of integration	[54]
Whole blood	Botulinum neurotoxin	Filtration <i>on-chip</i>	PDMS platform, immunosorbent assay, electrochemical detection	–	Sandwich format on agarose beads, high degree of integration	[55]
Serum	Human IgG	Filtration and dilution <i>off-chip</i>	Immunosorbent assay, LIF detection	Differentiation of normal from elevated IgG concentrations	Competitive format, biopassivated microchannels	[56]
Plasma (spiked)	Cardiac markers (CRP, Mb, cTnI and S100 α)	Dilution	PDMS microchip, immunosorbent assay, LIF detection	Analysis time: 10 min, LOD: 30 ng/mL	Sandwich format, micromosaic simultaneous determination	[57]
Serum (spiked)	B-type natriuretic peptide	Dilution	Glass/PDMS microchip, immunosorbent assay, SPR detection	Analysis time: 30 min, LOD: 5 pg/mL	Competitive format, <i>off-chip</i> incubation	[58]

3,4-dihydroxy-L-phenylalanine (L-DOPA), ascorbic acid (AA), and 3,4-dihydroxyphenylacetic acid (DOPAC). The modification of the electrode with cellulose-ssDNA prevents surface passivation and amplifies electrocatalytic activity through the interaction of the analyte and ssDNA. Cellulose regulates the solubility of ssDNA in aqueous media and ssDNA acts as an efficient promoter of a direct electron transfer reaction and/or the concentration of analytes on the electrode surface by means of the electrostatic interaction. The applicability of the system to DA analysis in the presence of AA in serum samples without treatment was demonstrated.

Schwarz's group [34] has developed a sensitive and selective method for the simultaneous determination of catecholamines and their cationic metabolites, without mutual interference, in mouse brain homogenates and human urine. They used a novel combination of additives (sodium dodecyl sulphate micelles, dendrimers forming a second pseudo-stationary phase, and borate complexation) to achieve complete separation on the chip and a carbon nanotube modified electrode as a detector to enhance sensitivity. Sample pre-treatment, made *off-chip*, is necessary in both cases. For urine, sample dilution in 0.2 M HClO₄ containing 0.1% of Na₂EDTA to prevent the oxidation of the analytes and filtration is required. The brain tissue was homogenated, centrifuged, and diluted in running buffer prior to injection.

Landers et al. [35] have demonstrated the possibility of high-throughput screening for several amino acids in urine samples using CE on a microchip format. Indirect fluorescence detection has been adapted to the electrophoretic microchip to provide a fast analysis of amino acids. Although the detection limits for indirect detection are lower than those achievable using fluorescence labeled analytes, the lack of sample preparation and simplified electrophoretic profiles make this technique attractive for many types of analysis. The analysis of urine samples required filtration and dilution of the sample to avoid the high ionic values derived from the presence of NaCl and other inorganic ions. Despite the lower signal-to-noise ratio obtained when compared to conventional CE (significantly longer capillary), the method allowed the quantitative determination of various amino acids in abnormal urine specimens.

Microchip CE-ED to measure uric acid in physiological urine samples was reported by Fanguy et al. [36]. The sample preparation for and the analysis of uric acid, with a clinically accepted enzymatic reaction, can be tedious. However, the proposed microchip system using electrochemical detection is able to evaluate single-filtered diluted urine samples in the expected range of the concentration of the analyte. Due to the low detection limit of the method, uric acid determination is accomplished in 50–75-fold dilution of the urine, without potential interference from other organic compounds with similar potential oxidation in the sample.

The groups of Wang and Henry reported the simultaneous analysis of different renal markers in urine samples using microchip CE [37,38]. In the first paper [37], creatine, creatinine, *p*-aminohippuric acid, and uric acid are determined by the coupling of an *on-chip* enzymatic assay, electrophoretic separation, and electrochemical detection. Hydrogen peroxide from

the enzymatic reactions of creatinine and creatine *on-chip* is separated from *p*-aminohippuric and uric acids, and the three species are amperometrically detected at the end-column detector. Urine samples were filtered and diluted prior to analysis. Henry et al. [38] reported the separation of these renal markers (creatine, creatinine and uric acid) using 30 mM borate buffer (pH 9.4) plus 1 mM SDS and their direct detection using pulse amperometric detection. The three analytes were determined in diluted and spiked urine samples, and the creatinine results were validated using a commercially available assay kit based on the Jaffe reaction.

Heightened levels of carbohydrates in biological fluids, mainly glucose, are especially relevant in diagnosing metabolic disorders. With this in mind, different microchip systems have been developed to determine carbohydrates in urine or blood samples. Wang and his group [39] reported an electrophoretic microchip to determine lactate and glucose in blood samples. This procedure combines the selectivity and amplification features of enzymatic assays with capillary-electrophoretic separation efficiency. Specifically, this microchip integrates electrophoretic separation of lactate and glucose in the separation channel, their post column reaction with the corresponding oxidases, and amperometric detection of the hydrogen peroxide liberated in the two separated product zones, as is illustrated in Fig. 1. Electrophoretic separation avoids potential electroactive interference, enhancing the advantages of enzyme flow-injection assays. Analyzed samples (serum and blood samples) must be filtered, centrifuged, and diluted in the running buffer *off-chip*.

Du et al. reported the electrophoretic separation and direct electrochemical detection of glucose on a PDMS/glass microchip [40]. The device includes copper microelectrodes manufactured onto the electrode plate by selective electrodeless deposition. Plasma samples from healthy and diabetic patients were previously diluted and filtrated, and glucose was determined in 120 s with a detection limit of 6 μ M.

Lactate and glucose were also determined using colorimetric enzyme assays from a different total microanalysis system (μ TAS) approach. Srinivasan et al. [41] reported an impressive "lab-on-a-chip architecture" for the automated analysis of glucose in physiological fluids. The *microfluidic* system they used is shown in Fig. 2. This lab-on-a-chip, based on electrowetting for microdroplet actuation, integrates sample injection elements, reservoirs, droplet formation structures, fluidic pathways, mixing areas, and optical detection sites all on the same substrate. Detection based on the colorimetric enzyme-kinetic method (Trinder's reaction) allows glucose to be determined in spiked serum, plasma, urine, and saliva samples with good concurrence to the reference method. Additionally, Karube's group [42] fabricated a compactly integrated flow cell with a chemiluminescent FIA system for determining lactate concentration in serum. The cell was made by micromachining techniques and mainly consists of a reactor, a mixing cell, and a spiral groove. Lactate was determined using lactate oxidase and was catalyzed by pyruvate and hydrogen peroxide. Subsequently, the hydrogen peroxide reacted with the luminol-ferricyanide reagent and the resulting chemiluminescent product was detected. Lactate concentration

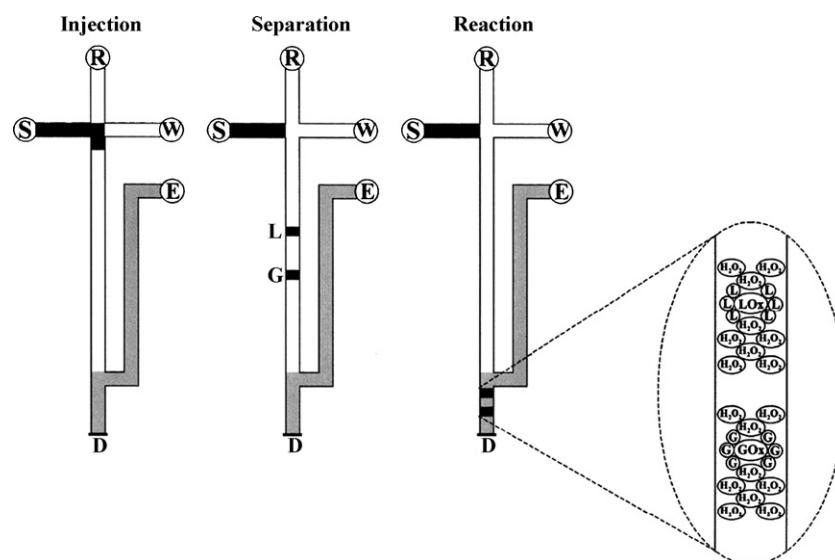


Fig. 1. Schematic of the CE-EC microchip, with the post-column enzymatic reaction, for the simultaneous measurement of glucose (G) and lactate (L). The individual analytical steps (injection, separation, reaction) are shown from left to right. Also shown (insert) is the post-column channel with the two hydrogen-peroxide zones. Reservoirs: (R) running buffer, (W) waste, (S) sample mixture, (E) 'reagent' (enzyme) solution containing glucose oxidase (GOx) and lactate oxidase (LOx); (D) the amperometric detector. The chip consisted of a sample (S), waste (W), and a running-buffer (R) reservoirs, connected through 5 mm long 'arms' to a four-way injection cross. A 77.7 mm-long separation channel followed the injection cross. An additional 77.2 mm-long channel (referred to as 'post-column') was joined the end of the separation channel, leaving a 10 mm channel for the enzymatic reaction before the detector. All channels were 50 μm wide and 20 μm deep (from reference [39], with permission).

was determined in undiluted spiked serum samples using this system.

In a similar fashion to ref. [41], Moon et al. [43] developed an integrated digital *microfluidic* chip for multiplexed proteomic sample preparation for MALDI-MS analysis. The system integrates the handling of solutions and reagents with electrowetting-on-dielectric (EWOD) actuation allowing the parallel processing of multiple sample droplets for high-throughput MALDI-MS. Processing four angiotensin peptide/urea samples demonstrated the suitability of the device for proteomic applications.

Spectrophotometric detection is not usually employed in *microfluidic* devices, mainly due to its low sensitivity; how-

ever, Caglar and co-workers [44] have developed a glass-PDMS microchip used to determine Ca^{2+} ions in serum samples taking reflectance measurements of arsenazo III immobilized on the surface of polymer beads. The beads were used at the detection point of the *microfluidic* sensor device with a fiber optic assembly for reflectance measurements. Despite potential interference from other ions, the microchip sensor allowed Ca^{2+} to be determined in untreated serum samples within the physiological range. In another approach, to overcome the usually low sensitivity associated with photometric microchips, Noda et al. [45] developed an optical path extension absorption-photometry microchip for highly sensitive hemoglobin measurements. In this device, the reflection in two 45° mirrors at each end of the

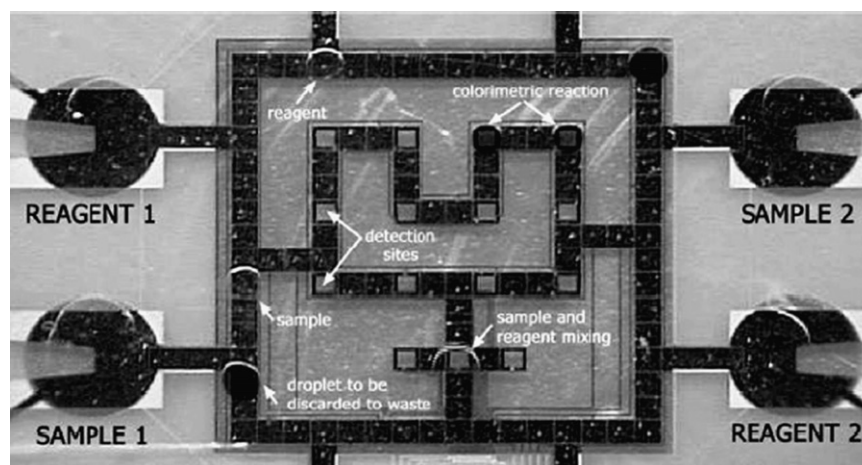


Fig. 2. Integrated digital microfluidic lab-on-a-chip for clinical diagnostics. This lab-on-chip, based on electrowetting for microdroplet actuation, integrates sample injection, on-chip reservoirs, droplet formation structures, fluidic pathways, mixing areas and optical detection sites, on the same substrate. Glucose is determined in spiked serum, plasma, urine and saliva as a proof of concept by colorimetric enzyme-kinetic method (Trinder's reaction) (from reference [41] with permission).

fluidic channel allows the propagation of light from the source to the detector along a longer optical path (5 mm). Blood samples that were previously diluted and to which the hemolysis chemical (cyanogen solution) were added were then photometrically measured allowing the determination of hemoglobin within the normal human concentration range (12–17 g/dl).

As is known, immunoanalytical systems have become one of the most widely used methods for detection and determination of a wide variety of analyte molecules in clinical diagnosis. Miniaturization of immunoassays onto microchip platforms combines the power of *microfluidic* devices with the high sensitivity and specificity of antigen–antibody interactions. The inherent reduction in diffusion distances for the species and the larger surface-area-to-volume ratio can increase the chances of the antigen and antibody of binding within the *microfluidic* channels. However, in a miniaturized immunoassay, particular points more important than in conventional systems can be considered. These include the effect of the capillary surface's chemical nature (non-specific binding), the methods of liquid transportation because of high flow resistance, special consideration for mixing procedures due to laminar flow, or specific detection principles because of the reduction in volume reaching the detector [23].

Taking into account the exceptional properties for miniaturizing CE together with the selectivity power of immunoassays, the coupling of both techniques (μ CEI) constitutes one of the most widely used approaches [46]. Herr et al. have developed *microfluidic* immunoassays based on gel electrophoretic separation and the quantization of bound and unbound species. In an earlier paper [47], direct immunoassay (non-competitive) and competitive formats were used in the detection of the tetanus antibody and the tetanus toxin, respectively, in spiked serum samples. *On-chip* photopatterned native PAGE gel was used for the electrophoretic separation of the fluorescently labeled C-fragment of the tetanus toxin with the specific antibody from its complex after an *off-chip* incubation step. Recently [48], the same researches improved their system by integrating sample pre-treatment *on-chip* (filtering, enrichment, mixing) with an electrophoretic immunoassay to determine the collagen-cleaving enzyme matrix metalloproteinase-8 (MMP-8) in saliva as a diagnostic fluid. Relevant clinical concentrations (LOD 130 ng/mL) are measured in less than 10 min. Samples must be centrifuged and previously diluted to 20 μ l before being loaded into the *microfluidic* system.

Exploiting the capability of CE to analyze multiple analytes in a single sample, Phillips and Wellner [49,50] designed an immunoaffinity CE to measure concentrations of up to 12 inflammatory neuropeptides and cytokines in muscle and cerebral spinal fluids. Using one reservoir of the microchip, Fab fragments from each of the specific antibodies were immobilized on their surface. After the incubation steps with sample and laser dye and the subsequent washing steps, the captured analytes were eluted and directed to the separation channel. In less than 160 s, 12 neuropeptides were resolved and determined in a 100 pL sample. Tissue fluid samples were previously passed through an ultra-micro dialyzer. In the same paper [50], an electrokinetic immunoassay was developed for the same analytes. However, 280 s were needed to resolve the immune complex of

the free-labeled antibody of 1 specific analyte, and higher LOD were obtained.

Kennedy et al. demonstrated the continuous monitoring of insulin secretion from live cells (islets of Langerhans) using a capillary electrophoresis competitive immunoassay [51]. Continuous sampling, on-line reaction, and electrophoretic separation of the fluorescein isothiocyanate-labeled insulin (FITC-insulin) and FITC-insulin-Ab-complex were accomplished in 30 s. A detection limit for insulin of 3 nM and continuous monitoring up to 30 min with no intervention were achieved.

Matsunaga's group [52] reported the use of a multichanneled matrix column coated with cation-exchange resin to separate immunocomplexes after a competitive binding event. Histamine is measured in 10 μ L of whole blood directly extracted from patients by an electrochemical flow immunoassay. Based on different isoelectric points of immunocomplexes, histamine-antibody and histamine-derivative-antibody, only the first is retained in the column. The conjugation of the specific antibody with ferrocene allows electrochemical detection of the immunocomplex in the eluent. Histamine concentration in the 200–2000 ng/mL range is measured in whole blood within 2 min.

The determination of cancer markers is crucial for an early diagnosis of the disease and immunoassay is considered an indispensable technique for determining small amounts of a tumor marker in serum. Different papers have reported using microchip-based immunoassays for cancer diagnosis [53,54,56]. Sometimes the electrophoretic separation of bound and unbound species is not suitable in the microchip format. An interesting alternative would be the integration of the widely used immunosorbent assay (ELISA), in which antigens or antibodies are fixed on a solid surface, onto a microchip known for its high sensitivity. Kitamori et al. [53] and Tsukagoshi et al. [54] have developed glass bead immunosorbent assays for the human carcinoembryonic antigen (CEA) and immunosuppressive acidic protein (IAP), respectively. In Kitamori's work, a sandwich immunoassay format together with thermal lens detection of colloidal gold-labeled antibodies is used. Serum samples from patients are assayed in 35 min with a determination limit of 0.03 ng/mL for CEA. Tsukagoshi uses chemiluminescence to detect unbound luminol-labeled analytes at the end of the microchannel where a microperoxidase catalyzes the hydrogen peroxide reaction. IAP spiked serum samples are determined in the relevant clinical range of concentrations within 2 min for each assay.

Also based on a sandwich ELISA format, where the specific antibody is immobilized on functionalized agarose gel beads, Moorthy et al. [55] developed a *microfluidic* platform for detecting botulinum neurotoxin directly from whole blood. Sample preparation by filtration, mixing and incubation with reagents were carried out on the device. Clinically relevant amounts of the toxin can be detected by measuring the color of an insoluble enzymatic product from the alkaline phosphatase reaction.

Elevated levels of monoclonal human IgG in serum may be linked to cancer. In this sense, Thormann's group [56] reported

a heterogeneous competitive human serum IgG immunoassay. Immunoglobulin G and Cy5-IgG, used as a tracer, compete in order to bind to a specific antibody immobilized on a biopassivated microchannel. Using Cy3-mouse IgG as an internal standard allows samples from patients with normal IgG serum levels (8–16 mg/mL) to be differentiated from patients with elevated IgG concentrations (>16 mg/mL).

A sophisticated approach is reported by Delamarche's group [57] in which different cardiac markers are simultaneously detected. They combine *microfluidic* networks, in which the flow of liquids is driven by capillary forces (self-regulating), and micromosaic immunoassays based on patterning lines to capture antibodies exposed to solutions of analytes. Using secondary fluorescently labeled antibodies, a micromosaic of fluorescent zones reveals the binding event in a single imaging step. In this case, four cardiac markers – C-reactive protein (CRP), myoglobin (Mb), cardiac troponin (cTnI), and S100 α – are simultaneously determined in spiked plasma samples (1 μ L volume) within 10 min.

Recently, Niwa et al. [58] reported a *microfluidic* device combined with a portable surface plasmon resonance sensor system (SPR) to determine the cardiac marker B-type natriuretic peptide (BNP) in spiked serum samples. After competitive immunoreaction takes place, the thiol compound generated by the enzymatic reaction with the trapped labeled antibody was accumulated on a thin gold film located in the microchannel. The extension of the chemisorption of thiol molecules can be monitored by the SPR angle shift and related to BNP concentration. A concentration range of 5 pg/mL to 100 ng/mL covers the usual levels of BNP in blood.

3. Real environmental and related sample analysis on *microfluidics*

The development of portable, robust, and accurate analytical systems to monitor environmental compounds such as contaminants, explosives, chemical warfare agents, inorganic and organic ions is a demand of modern society. Portability allows analyses to be carried out outside of the laboratory, preventing or minimizing the risk of contaminating the sample and leading to a faster response time at a lower cost. The ability to use portable equipment is an intrinsic characteristic of *microfluidic* systems, which is why they are an excellent response to this analytical demand.

Sample introduction, complexity of the sample matrix, and the low concentration of the chemical species in the environment are the possible challenges of using *microfluidic* devices for environmental analysis.

There are excellent bibliographic reviews in the literature about *microfluidic* devices for environmental analysis [59,60], but not reviews are found on the analysis of real samples and their particular challenges.

In this section, the most relevant papers in the literature that use *microfluidic* devices for environmental analysis have been classified and discussed according to the kind of analytes studied: warfare agents and explosives, and pollutants and ions of environmental interest.

Table 2 summarizes the most relevant information regarding the analysis of the main compounds of environmental significance in real samples using miniaturized devices.

Wang and co-workers have developed a method for determining thiol-containing degradation products of V-type nerve agents in water samples [61]. The detection of three of these compounds was achieved in only 4 min, though only in a water sample spiked with its corresponding standards. The microsystem consists of a glass capillary electrophoresis microchip with amperometric detection (screen-printed carbon ink electrode). A derivatization of the compounds with o-phthalaldehyde was necessary, which was developed both *on-* and *off-chip*. When the process was performed *on-chip*, the complete reaction between derivatization agent and compounds was not achieved, resulting in decreased sensibility. With the same microsystem [62], three organophosphate nerve agents were detected in 140 s from a river water sample spiked with those analytes. In both cases [61,62], the LODs were in micromolar range, but this seems to be insufficient for real applications. Better LODs were obtained by the same research group using contactless conductivity as its detection mode [63]. A detection limit within the nanomolar range was achieved for the determination of three organophosphate nerve agent degradation products from a river water sample (spiked with standards). In this paper, a disposable PMMA microchip with integrated electrodes was used with excellent results (analysis time 130 s).

Luong et al. were able to determine trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene in soil and groundwater samples by MEKC-EC on a glass microchip and using a gold wire as electrode [64]. The analysis time was 4 min and LODs were below the micromolar range. The accuracy of the method was validated by comparison with the LC procedure of the US EPA (method 8330). For the soil sample, an extraction step *off-chip* was necessary. In all papers, the water samples were filtered *off-chip*.

Wang and co-workers have also separated and detected three different groups of pollutants (nitrophenols, aromatic amines, and chlorophenols) by capillary electrophoresis on a glass microchip with amperometric detection using river and groundwater samples. In this case, the detected compounds were previously added. Five nitrophenol derivatives were detected in 120 s with a glassy carbon electrode [65], three aromatic amines (4-aminophenol, 2-aminonaphthalene, and o-aminobenzoic acid) in 150 s with a boron-doped diamond thin-film detector [66], and phenol and three chlorophenols in 120 s with a carbon SPE modified with gold [67]. In these papers, the LODs were within the micromolar range and accuracy was not evaluated. The samples were filtered before the analysis.

Garcia et al. obtained good results in their analysis of environmental samples using a PDMS microchip with pulse amperometric detection (PAD). Levoglucosan (the largest single component of the water extractable organics in smoke particles) was determined in smoke particles in only 1 min [68]. The results obtained with this method are in accordance with those obtained with GC/MS. The aerosol particles were collected and extracted *off-chip* and *off-line*. The LOD achieved (17 μ M) was adequate for the analysis of smoke samples but insuffi-

Table 2
Microfluidics (capillary-electrophoresis microchip) in environmental analysis

Sample	Analytes	Sample treatment	Microfluidic system	Detection mode	LOD (μM)	Analysis time (s)	Accuracy evaluation	Ref.
Tap and river water (spiked)	2-(Dimethylamino) ethanethiol, 2-(diethylamino) ethanethiol, 2-mercaptoethanol	Derivatization agent (o-phthalaldehyde) in presence of Valina, <i>off- and on-chip</i>	Glass microchip, CZE	Amperometry carbon ink screen-printed electrode	6–11	240	–	[61]
River water (spiked)	Paraoxon methyl parathion, fenitrothion	Filtration, <i>off-chip</i>	Glass microchip, MEKC	Amperometry, carbon ink screen-printed electrode	0.9–3.8	140	–	[62]
River water (spiked)	Methylphosphonic acid, ethylphosphonic acid, isopropylphosphonic acid	Filtration, <i>off-chip</i>	PMMA microchip, CZE	Contactless conductivity	0.005–0.006	130	–	[63]
Soil and groundwater	Trinitrotoluene, 2,4-dinitrotoluene, 2,6-dinitrotoluene	Extraction, centrifugation and filtration, <i>off-chip</i>	Glass microchip, MEKC	Amperometry, gold electrode	0.48–0.88	240	Comparison with LC procedure (method 8330) US EPA	[64]
Groundwater (spiked)	2-Nitrophenol, 3-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, 2-methyl-4,6 dinitrophenol	Filtration, <i>off-chip</i>	Glass microchip, MEKC	Amperometry, glassy carbon electrode	7–30	120	–	[65]
River water (spiked)	4-Aminophenol, 2-aminonaphthalene, o-aminobenzoic acid	–	Glass microchip, CZE	Amperometry, boron-doped thin-film detector	1.3–2	150	–	[66]
River water (spiked)	Phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,3-dichlorophenol	Filtration, <i>off-chip</i>	Glass microchip, CZE	Amperometry, carbon ink screen-printed electrode modified with gold	1–2	120	–	[67]
Smoke particles	Levogluconan	Aerosol particles collection and extraction <i>off-chip</i>	PDMS microchip, CZE	Pulse amperometric detection Gold electrode	17	60	Comparison with GC/MS	[68]
City water (spiked), medicines	Phenol 4,6-dinitro-o-cresol pentachlorophenol	–	PDMS microchip, CZE	Pulse amperometric detection, gold electrode	0.9–2.2	120	Respect declared content (label medicine)	[69]
River water	DOC (dissolved organic carbon)	Derivatization with isothiocyanate and filtration, <i>off-chip</i>	Glass microchip, CZE	LIF	–	120	–	[70]
Electroplating sludge reference material	Pb(II)	Extraction, centrifugation and filtration, <i>off-chip</i>	PDMS microchip, sensor	LIF	0.011	–	Respect reference material	[71]
Water mimetic solution	Cr(III)	EDTA to remove interferences, <i>off-chip</i>	PDMS-glass microchip, FIA (mixer/reactor system)	Chemo luminescence (luminol-H ₂ O ₂)	0.1	60	Recovery	[72]
River, pond and rain water	Sulfite, nitrite	Derivatization agent, N-(9-acridinyl) maleimide, 2,3-diamino naphthalene, <i>on-chip</i>	PDMS-glass microchip, FIA	LIF	0.4–1	900	Recovery	[73]
Plexiglas surface contaminated	Cd(II), Pb(II), Co(II), Ni(II)	Chelating agent 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfo) propylaminophenol, C ₁₈ silica column, <i>off-chip off-line</i>	Glass microchip, non-aqueous CZE	Colorimetric	0.003–0.053	80	Recovery	[74]
River, tap, mineral water	Nitrite, fluoride, phosphate	Preconcentration and elimination of interferences, <i>on-chip on-line</i>	PMMA microchip, ITP-CZE column coupled	Conductivity	0.5–0.7	360	–	[75]

cient for atmospheric samples. In another paper [69], three of the most important pollutants (phenol, 4,6-dinitro-*o*-cresol, and pentachlorophenol) were detected in a city water sample spiked with the corresponding standards. The LODs were within the micromolar range and accuracy was evaluated by determining phenol in two medicines with labeled content. Sample treatment was not necessary.

An original method was developed for evaluating the environmental index DOC (dissolved organic carbon) [70]. The microsystem consists of a glass capillary electrophoresis microchip with LIF as its detection mode. Derivatization with a labeling agent, fluorescein isothiocyanate, was needed (overnight). A sample of river water was analyzed, but the results were not compared with other methods.

Anions and cations of environmental interest have also been explored. Chang et al. fabricated a sensor to monitor Pb^{2+} in environmental samples [71]. This microdevice was made on PDMS and contained a network of *microfluidic* channels that were fluidically coupled via a nanocapillary array interconnect (NAI) and used LIF as its detection mode. This sensor's selectivity was achieved using a lead-specific DNAzyme constructed by the authors which possessed a fluorophore fragment. In the presence of Pb^{2+} , the substrate DNA was cleaved, resulting in the release of fragments and a concomitant increase in fluorescence, as is illustrated in Fig. 3. Accuracy was evaluated with an electroplating sludge reference material, but no further samples were analyzed. An excellent LOD was achieved (11 nM).

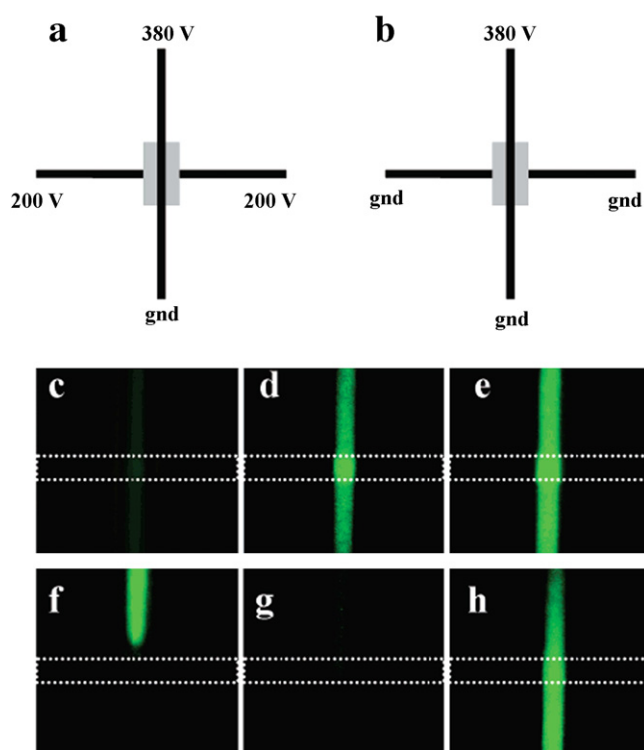


Fig. 3. Electrical bias configurations for fluidic control of lead and DNAzyme within an NAI/microfluidic device: (a) on state and (b) off state. Temporal sequence of fluorescence images at the intersection of the crossed microchannels (c–h) (from reference [71] with permission).

Two different research groups have used capillary electrophoresis microchips as FIA systems. In the first paper [72], Cr (III) was detected by chemiluminescence in a water mimetic solution in 1 min. The catalytic property of Cr (III), the result of the reaction between luminol and H_2O_2 , was taken advantage of to determine this cation. All parameters of the mixer/reactor microsystem were optimized and *off-chip* sample treatment with EDTA was needed to remove interferences (other metal cations). In the second paper [73], sulfite and nitrite were detected by FIA-LIF in different water samples, but the use of two derivatization agents was needed (*on-chip*). LODs within the micromolar range and a huge analysis time (15 min) were obtained. The accuracy of both methods was evaluated by recovering the standards added in the studied samples. Deng and Collins separated and detected four metallic cations (Cd^{2+} , Pb^{2+} , Co^{2+} , Ni^{2+}) using a non-aqueous capillary electrophoresis microchip with colorimetric detection on a contaminated Plexiglas surface prepared by the authors [74]. Sample treatment was carried out *off-chip* and *off-line* and consisted of extraction with a chelating agent. The extract was then preconcentrated in a C_{18} silica microcolumn, and, finally, the sample was introduced onto the microchip achieving LODs within the nanomolar range.

Bodor et al. have determined three anions (NO_2^- , F^- , PO_4^{3-}) in river, tap, and mineral water on a PMMA column-coupling microchip with conductivity detection [75]. The illustration of the microdevice used is shown in Fig. 4. The microchip consists of two coupled columns: The first is used as an isotachopheresis (ITP) column and the second as a CZE column. In the ITP stage, the sample was preconcentrated and cleaned up and, in the CZE stage, the anions were separated. Minimal sample treatment *off-chip* was needed (filtration and degassing). LODs were within the micromolar range and the accuracy of the method was not evaluated.

4. Real food sample analysis on *microfluidics*

The state of the art of food analysis on CE microchip platforms has recently been revised [76]. In food analysis, sensitivity and selectivity are also the major drawbacks. In contrast to biochemical analysis where the very specific reagents (immunoassay/enzymes) are frequently used and dramatically improve the selectivity in the analysis, in food analysis, the use of bio-reagents is obviously less developed and sample preparation is always needed involving complex sample preparation protocols and analyte separation with advanced detection schemes.

In food analysis, *microfluidics* are also now emerging since, as was mentioned before, sample preparation integration is less developed due to its inherent complexity. The *microfluidics* used are mainly CE microchips applied to both liquid and solid samples. The first approaches focused on exploring fast separation and suitable detection routes for prominent analytes with food significance. In all cases, the sample preparation step is carried out *off-chip* and microchips are merely used as fast analytical separation systems. In consequence, the first “real samples” analyzed are still *easy*, such as nutraceuticals and dietary supplements.

Table 3
Microfluidics (capillary-electrophoresis microchip) in food analysis

Sample	Analytes detected in food sample	Sample preparation	Microfluidic approach	Analytical characteristics	Remarks	Ref.
Red wine (one)	Chlorogenic, vanillic	Dilution and filtration, <i>off-chip</i>	Glass CE microchip, electrochemical detection (SPE electrode)	Separation time (<300 s), good precision, less than 7% in peak areas, external calibration ($r > 0.99$), LOD = 10 μM	Use of red wine (high polyphenolic content), use of different oxidation potentials during wine analysis	[77]
Beverages	Inorganic cations (water, juices, beer and milk): ammonium, potassium, calcium, sodium, magnesium. Inorganic anions (water): chloride, nitrate, sulfate	Dilution and filtration, <i>off-chip</i>	PMMA microchip, contactless conductivity	Separation time (<40 s), LODs (90–150 $\mu\text{g/L}$)	High degree of integration, quantitative analysis is given for all analytes	[78]
Vitamin C tablets	Vitamin C	Power and filtration	PMMA	Separation time (<50 s)	Quantitative analysis is given for all analytes	[79]
Soft drinks	Preservatives, benzoate, sorbate	<i>Off-chip</i>	Simple cross, capacitively coupled contactless conductivity	Good precision (less than 4%), external calibration ($r > 0.99$), LODs 3–10 mg/L	Comparison with CE conventional	
Wine, beer	Tryptamine, tryptophan, tyramine	Filtration, <i>off-chip</i>	Miniaturized CE-ED, electrochemical detection (Cu electrode)	Separation time (<360 s), good precision (RSD 3.6%), LODs 0.6–1.5 μM	Home-made miniaturised CE system	[80]
Green tea extract (nutraceutical)	(+) Catechin, epigallocatechin gallate, (–)epicatechin, epicatechin gallate	Solid–liquid extraction and filtration, <i>off chip</i>	PDMS CE, microchip, electrochemical detection	Separation time (<300 s), good precision, less than 7% in peak areas, external calibration ($R^2 > 0.99$), LODs 8–10 μM	Use of suited real sample (high concentration), quantitative analysis is given for all analytes	[81]
Pear pulps and commercial juices	Arbutin, ascorbic acid	Juice filtration or solid–liquid extraction and filtration, <i>off-chip</i>	Glass CE microchip, electrochemical detection	Separation time (<200 s), good precision, less than 7% in peak areas, external calibration ($R^2 > 0.99$)	Separation of target antioxidant couples	[82]
Formulations	Water-soluble vitamins: pyridoxine B6, vitamin C, folic acid	Solution and filtration, <i>off-chip</i>	Glass CE microchip, electrochemical detection	Separation time (<130 s), high accuracy (errors less than 7%)	Calibration and determination in less than 350 s, quantitative analysis is given for all analytes	[83]
Vanilla pod and sugars	Vanillin, ethyl vanillin	Extraction and maceration, <i>off-chip</i>	Glass CE microchip, electrochemical detection	Separation time (<200 s), good precision less than 7% in peak heights	Detection of frauds	[84]
Green tea	Arg, The, Gly	Extraction and filtration and derivatization of aminoacids, <i>off-chip</i>	PMMA microchip with LIF detection	Separation time (120 s), precision chip-to-chip 3%	Derivatization is not selective to aminoacids, selectivity is improved by removing the high content of catechins in tea samples, validation of the method using HPLC	[85]
Natural extract	Total isoflavones	Extraction and filtration	Valveless microsystem	Analysis time (60 s)	Calibration integrated.	[86]
Dietary supplements		<i>Off-chip</i>			Accuracy is evaluated using a secondary standard (error less than 7%)	
Vegetables, fruits and meats	Nitrite	Extraction and 732-cation resin column and, filtration, <i>off-chip</i>	PMMA microchip (μFIA), chemiluminescence detection	Separation time (<120 s), good precision less than 4.1%, external calibration ($r > 0.99$), LOD = 4 $\mu\text{g/L}$ hydrodynamic flow	Validation respect AOAC official method (Griess method), interferents (Fe^{2+} , Ni^{2+} , V (V), Cu^{2+} , uric acid and vitamin C)	[87]
Milk (spiked)	Staphylococcus enterotoxin B	Filtration, <i>off-chip</i>	PDMS microchip, fluorescence detection	Sandwich-type immuno assay, fluorescently labelled antibody, LOD = 0.5 ng/ml, hydrodynamic flow	–	[88]
Baby powder, milk, sucrose (spiked)	Botulinum neurotoxin A	Filtration, <i>off-chip</i>	Polycarbonate ELISA-chip, colorimetric detection	Sandwich-type immuno assay, cross-flow immuno-chromatography, horseradish peroxidase, LOD = 2 ng/ml	Five-fold enhancing of LOD respect the kit commercially established	[89]
Infant formula	Folic acid	Filtration, <i>off-chip</i>	Commercial polyimide immuchip for ELISA, electrochemical detection (Gold electrode)	Competitive immuno assay, hydrodynamic flow, analysis time = 5 min, R.S.D. 7% alkaline phosphatase	Validation respect reference method (microbiological assay)	[90]

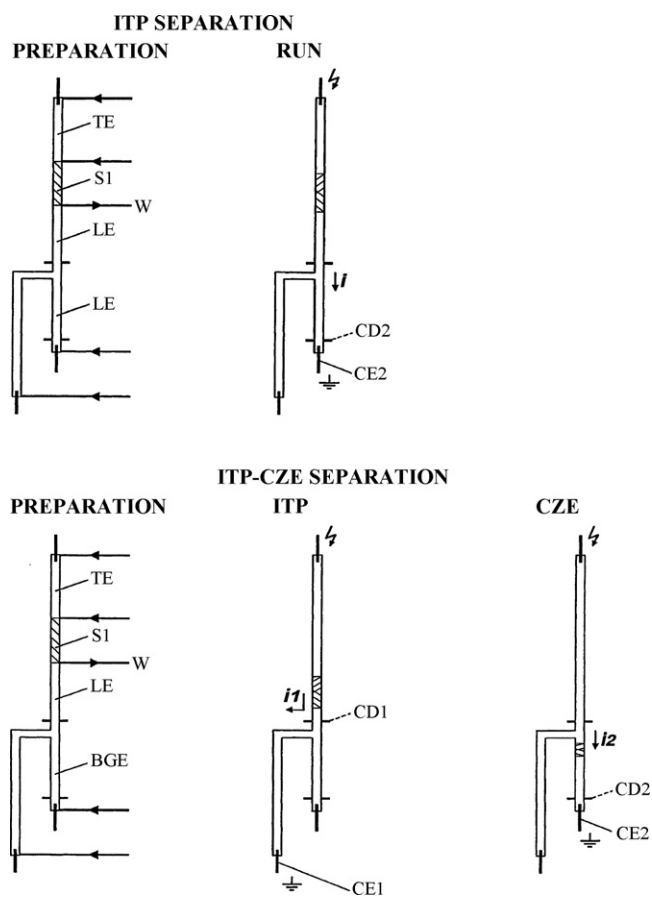


Fig. 4. Schemes of the ITP (the tandem-coupled separation channels) and ITP-CZE separation modes as employed in the separations of inorganic anions on the CC chip (from reference [75], with permission).

Table 3 lists the relevant information about food analysis on, primarily, CE-microchips and other *microfluidic* systems. Next, we are going to discuss the relevant keys to understanding the present role of *microfluidics* in food analysis.

Liquid samples, as opposed to solid samples, require minor sample preparation, and they have been studied by direct injection using both amperometry and conductimetry. This way, two single phenolic acids, vanillic and chlorogenic acid, were detected in red wine samples in less than 300 s using a CE microchip with amperometry detection [77]. The overall protocol involves simple sample preparation *off-chip* (filtration and dilution in an electrophoretic buffer), and the wine sample was directly injected onto the microchip. Selectivity and sensitivity was checked using different oxidation potentials as a strategy to obtain relevant information using the wine sample. As expected, lower potentials provided greater selectivity while higher ones led to increased sensitivity. Red wines are very complex and a lot of polyphenols are present since red wine is one of their most important sources. Probably, the detection of just acid structures was due to the fact that these compounds are prominent, while flavonoids did not show any interference because of the dilution performed.

The elegant approach, based on the use of a universal detector (*contactless* conductivity) coupled to a microchip in the analysis of liquid samples where selectivity is achieved by microchip

separation, is illustrated in the papers of Hauser's group [78]. These papers strategically combine the minor sample preparation requirements of this kind of samples (just dilution and filtration) with the inherent characteristics and advantages of (*contactless*) conductivity. Indeed, water, wine, beer, and fruit juice samples were simply diluted in electrolyte solution. Separations involving inorganic cations (ammonium, potassium, calcium, sodium, and magnesium), inorganic anions (chloride, nitrate, sulphate), and the simultaneous analysis of up to 12 inorganic and organic anions (oxalate, tartrate, malate, citrate, succinate, acetate, lactate) in less than 100 s were obtained. In addition, a detailed evaluation of the methods including reliable quantitative data was given indicating the power of these microdevices. The inherent advantages of miniaturization for food analysis using both liquid (beverages) and solid samples (vitamin C tablets) were also demonstrated illustrating the separation of commonly used preservatives such as benzoate and sorbate, and vitamin C using a PMMA microchip coupled with *contactless* conductivity [79].

Ye et al. [80] fabricated a miniaturized CE-ED system cutting a conventionally fused silica capillary and gluing it onto a Plexiglas plate. This system was used in the determination of three bioactive amines (tryptamine, tryptophan, and tyramine) in wine and beer. Good reproducibility (3.6%), analysis time (6 min), and LOD (μM range) were obtained.

Solid samples are very complex, and tedious *off-chip* extraction processes are always required before analysis. An analysis of five flavan-3-ols (catechins and derivatives) in commercially available green tea extract was performed using a PDMS microchip with MEKC and pulsed amperometric detection by Henry's group [81]. The detection of (+)-catechin, epigallocatechin gallate, (–)-epicatechin, and epicatechin gallate was achieved in less than 3 min in tea extracts (functional food). In addition, two more unidentified peaks were detected during analysis (probably attributable to other polyphenols). A quantitative analysis was performed and good accuracy was demonstrated. Sample preparation was still complex and *off-chip* (methanol–water, stirred extraction, and filtration), and the sample matrix was still “easy” since a nutraceutical was assayed containing high analyte concentrations; however, this paper was relevant since it clearly demonstrated the excellent analytical performance of these microsystems during sample quantitation.

Escarpa's group has proposed different strategies in the analysis of food samples involving natural antioxidants (polyphenols and vitamins) using single channel microchip electrochemistry platforms with electrochemical detection [82,83]. Polyphenolic analysis is very complex because many compounds could contribute to the complexity of the electropherogram obtained (as was also illustrated in Henry's papers). Although fast detection of prominent natural antioxidants including ascorbic acid and polyphenols was obtained in less than 200 s, the analysis of pear samples was very complex and only arbutin in some pulps and ascorbic acid in juice (where up to four peaks were observed) was really detected [82]. Recovery studies using blank samples were carefully performed for both analytes in order to confirm their provisional identification. The detection of phlorizdin (the *fingerprint* analyte of apples and

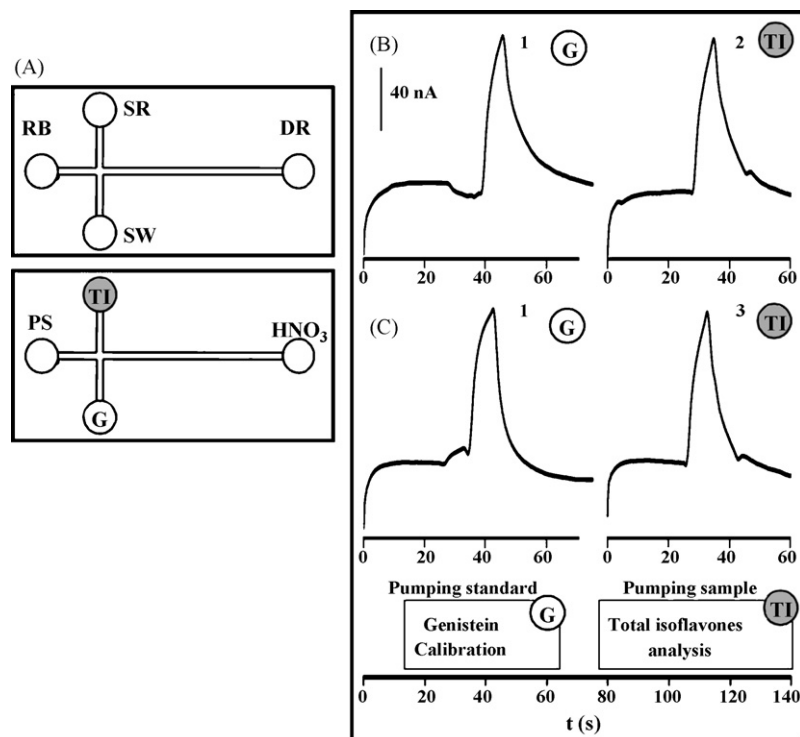


Fig. 5. Microchip layout used with classic and proposed terminology (A): genistein reservoir (G), total isoflavones reservoir (TI), pumping solution reservoir (PS). Calibration (using genistein G) and analysis signals obtained for total isoflavones (TI) in the reference material (B) and soy supplements (C) using the proposed integrated strategy. Conditions: 100 mM genistein peak (G, peak 1), total isoflavones peak [TI, peak 2 (reference material), peak 3 (dietary supplement)] (from reference [86], with permission).

unexpected in pears) in the presence of arbutin (the *finger-print* of pears) was also noticed, demonstrating the possibility of adulterations. Real detection and the quantitative analysis of water-soluble vitamins has been reported in several formulations containing one or more of the analyzed vitamins at different concentration levels in less than 130 s [83]. The amount of each vitamin declared by the manufacturer (per capsule) on the label closely matched the values obtained using the microchip protocol (relative error ranging from 2 to 9%). The well-known problems associated with the inherent features of electrokinetic injection (modification of ionic strength, compatibility between buffers and solvents/matrices) made quantitation more difficult than expected in those samples with high mineral salt content.

Very recently, an approach for detecting fraud in vanilla samples based on the analysis of ethyl vanillin and vanillin was proposed [84]. Vanillin was really detected in vanilla beans (*Vanilla planifolia* and *Vanilla tahitensis*), being this compound the prominent compound in vanilla extracts and which confirms their authenticity. In addition, ethyl vanillin was really detected in certain vanilla sugars indicating the non-natural origin of the vanilla flavor used since the presence of ethyl vanillin is unambiguous proof of fraud. On the contrary, vanillin was really detected in the other sugar samples which did not necessarily indicate a natural vanilla origin because, in and of itself, the presence of vanillin is not unambiguous proof of the natural origin. A detailed quantitative analysis is given and important differences in the quantitative levels of vanillin were found between

natural and adulterated samples revealing the possibilities of the method in control analysis.

Three prominent green tea amino acids (theanine (The), arginine (Arg), and glycine (Gly)), previously extracted and derivatized *off-chip*, were separated onto a PMMA microchip format using LIF detection in less than 2 min [85]. While sensitivity was not a challenge because of the detection route used, the selectivity of the analysis was improved by removing catechins, since the derivatization scheme involved both kind of compounds, amino acids and catechins.

Recently, methodological calibration and analysis on board a planar *microfluidic* device to determine *total* isoflavones in soy samples was proposed [86]. The strategy, in connection with microchip layout, is shown in Fig. 5. Accuracy (systematic error below 6%) is demonstrated for the first time using these microsystems with a secondary standard from the Drug Master File (SW/1211/03) as *reference* material. Ultra-fast calibration and analysis of total isoflavones in soy samples was successfully integrated, taking only 60 s each, notably enhancing the analytical performance of these microdevices with an important decrease in overall analysis times (less than 120 s) and with an increase in accuracy by a factor of 3. In addition, the method was applied to the analysis of soy tablets with acceptable accuracy.

He et al. [87] have developed a method to determine nitrite in different food samples (vegetables, fruits, and meats) using a PMMA chip as a μ FIA system with chemiluminescence detection (luminol-ferricyanide system). Exhaustive extraction and sample treatment (732-cation resin column) was needed per ana-

lyzed solid sample. Good analytical performance was achieved with a reproducibility of 4.1%, a detection limit of 4 µg/l, and an analysis time under 2 min. An interference study was performed discovering that Fe²⁺, Ni²⁺, V (V), Cu²⁺, uric acid, and vitamin C interfered with the analysis. Nevertheless, the method was compared with the AOAC official method (Griess method) with excellent results.

To improve selectivity and sensitivity, some immunoassays have been developed on *microfluidic* devices for food analysis as an alternative strategy. Cheng et al. [88] determined staphylococcal enterotoxin B in spiked dry milk using a PDMS chip with fluorescence detection. Sandwich-type immunoassays were developed utilizing the chip as a chamber reaction. In the first step, the chip surface was functionalized with biotinylated antibodies. In the second step, the sample was hydrodynamically introduced onto the chip and, then, the fluorescently labeled secondary antibodies were added. Finally, the chip was imaged and quantified on a fluorescence scanner. An excellent LOD was achieved with this method (0.5 ng/ml).

Botulinum neurotoxin A [89] was detected in spiked food samples (infant formula, milk, sucrose) on a polycarbonate ELISA-chip with colorimetric detection. A cross-flow immuno-chromatographic assay was developed in which a sandwich-type immunoassay with an enzyme-linked antibody was performed vertically, and a flow of the enzyme substrate revealed the presence of Botulinum neurotoxin A horizontally. An excellent LOD (2.0 ng/ml) was achieved, enhancing five-fold the LOD established by the commercial kit.

Another interesting ELISA on *microfluidic* devices was performed to determine folic acid in infant formula [90]. A commercial polyimide Immuchip ELISA with eight channels and hydrodynamic flow was used. In this case, a competitive assay between free antigens (sample analyte) and fixed antigens on the surface of the chip was performed and the product of the enzyme reaction was monitored by electrochemical detection ($E = 250$ mV). Excellent analytical performance (reproducibility 7%, LOQ 2 ng/ml, and analysis time 5 min) was obtained. The method was validated with the reference method (microbiological assay).

5. Conclusion and future prospects

Nowadays, and strictly speaking, there are few papers that deal with the analysis of real samples using *microfluidic* devices regardless of the application area. It clearly shows the enormous difficulties that the analysis of real samples presents on a microscale. Sensitivity and selectivity are the main challenges to developing a true “lab-on-a-chip,” with their improvement in terms of the use of ultra-sensitive detectors and the integration of sample treatments on the *microfluidic* devices being the main requirements. Moreover, additional efforts have to be made towards the validation of the methods to demonstrate the reliability of *microfluidic* systems. In spite of these drawbacks, incredible analysis times, low sample and reagent consumptions, and interesting applications involving clinical, environmental, and food analysis have been published.

No doubt exists that performing sample preparation *on-chip*, discovering novel ultra-sensitive detection routes, and systematically using *smart* bioreactives and nanotechnology are the keys to defining the success and incorporation of *microfluidic* devices in our laboratories.

Congratulations

I had the privilege of knowing Dr. Wang in 2001 during my *post-doc* position at NMSU and I was highly impressed by the quality of this person. I soon realized that not only was he an extraordinary scientist but a unique, talented human being. I firmly believe that we have before us a scientist with enormous creativity and a novel vision. I will never find the right words to say thanks!

Thank you very much, Dr. Wang!

What can we expect from words?

At times, they hardly sing.

But today they are here to tell us

That in your intelligence you are great

That you belong to that group of great men,

Different men, who live awake because they have a dream.

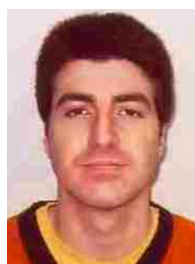
Mentor of mine,

My heart sings something about you

I find you in my memory

Like a friend that I have loved.

(Poem written by Alberto Escarpa for Dr. Wang in his 60th birthday)



Agustín González



Alberto Escarpa

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References

- [1] A. Manz, N. Graber, H.M. Widmer, *Sens. Actuators B* 1 (1990) 244.
- [2] G.W. Whitesides, *Nature* 442 (2006) 368.
- [3] D. Janasek, J. Franzke, A. Manz, *Nature* 442 (2006) 374.
- [4] O. Geschke, H. Klank, P. Tellesmann (Eds.), *Microsystem Engineering of Lab-on-a-Chip Devices*, WILEY-VCH Verlag & Co., KgaA, Weinheim, 2004.
- [5] A. Rios, A. Escarpa, M.C. Gonzalez, A.G. Crevillén, *Trends Anal. Chem.* 25 (2006) 467.
- [6] M.A. Schwarz, P.C. Hauser, *Lab Chip* 1 (2001) 1.
- [7] W.R. Vandaveer, S.A. Pasas, R.S. Martin, S.M. Lunte, *Electrophoresis* 23 (2002) 3667.
- [8] J. Wang, *Electroanalysis* 17 (2005) 1133.
- [9] M. Pumera, A. Merkoçi, S. Alegret, *Trends Anal. Chem.* 25 (2006) 219.
- [10] P.J. Viskari, J.P. Landers, *Electrophoresis* 27 (2006) 1797.
- [11] A. van der Berg, P. Bergveld, *Lab-on-a-Chip* 6 (2006) 1266.
- [12] J. Lichtenber, N.F. de Rooij, E. Verpoorte, *Talanta* 56 (2002) 233.
- [13] S.K. Mohanty, D. Kim, D.J. Beebe, *Electrophoresis* 27 (2006) 3772.
- [14] C.K. Fredrickson, Z.H. Fan, *Lab Chip* 4 (2004) 526.
- [15] G.T.A. Kovacs, *Micromachined Transducers Sourcebook*, McGraw Hill, New York, 1998.
- [16] R.H. Liu, J. Yang, R. Lenigk, J. Bonanno, P. Grodzinski, *Anal. Chem.* 1 (2004) 1824.
- [17] K.M. Horsman, J.M. Bienvenue, K.R. Blasler, J.P. Landers, *J. Forens. Sci.* 52 (2007) 784.
- [18] J.A. Fruetel, R.F. Renzi, V.A. Vandernoot, J. Stamps, B.A. Horn, J.A. West, S. Ferko, R. Crocker, C.G. Bailey, D. Arnold, B. Wiedenman, W.Y. Choi, D. Yee, I. Shokair, E. Hasselbrink, P. Paul, D. Rakestraw, D. Padgen, *Electrophoresis* 26 (2005) 1144.
- [19] P.N. Floriano, N. Christodoulides, D. Romanovicz, B. Bernard, G.W. Simmons, M. Cavell, J.T. McDevitt, *Biosens. Bioelectron.* 20 (2005) 2079.
- [20] L.J. Kricka, P. Fortina, *Clin. Chem.* 48 (2002) 1620.
- [21] A.J. Tudos, G.A.J. Besselink, R.B.M. Schasfoort, *Lab Chip* 1 (2001) 83.
- [22] E. Verpoorte, *Electrophoresis* 23 (2002) 677.
- [23] U. Bilitewski, M. Genrich, S. Kadow, G. Mersal, *Anal. Bioanal. Chem.* 377 (2003) 556.
- [24] D.D. Cunningham, *Anal. Chim. Acta* 429 (2001) 1–18.
- [25] A.J. de Mello, N. Beard, *Lab Chip* 3 (2003) 11N.
- [26] S. Thorslund, O. Klett, F. Nikolajeff, K. Markides, J. Bergquist, *Biomed. Microdev.* 8 (2006) 73.
- [27] S.F.Y. Li, L.J. Kricka, *Clin. Chem.* 5 (2006) 37.
- [28] B. Baena, A. Cifuentes, C. Barbas, *Electrophoresis* 26 (2005) 2622.
- [29] W.R. Vandaveer, S. Pasas-Farmer, D.J. Fischer, C.N. Frankenfeld, S.M. Lunte, *Electrophoresis* 25 (2004) 3528.
- [30] B.H. Weiller, L. Ceriotti, T. Shibata, D. Rein, M.A. Roberts, J. Lichtenberg, J.B. German, N.F. de Rooij, E. Verpoorte, *Anal. Chem.* 74 (2002) 1702.
- [31] L. Ceriotti, T. Shibata, B. Folmer, B. Weiller, M.A. Roberts, N.F. de Rooij, E. Verpoorte, *Electrophoresis* 23 (2002) 3615.
- [32] J.A. Lapos, D.P. Manica, A.G. Ewing, *Anal. Chem.* 74 (2002) 3348.
- [33] M. Johirul, A. Shiddiky, R. Kim, Y. Shim, *Electrophoresis* 26 (2005) 3043.
- [34] M. Vlckova, M.A. Schwarz, *J. Chromatogr. A* 1142 (2007) 214.
- [35] N.J. Munro, Z. Huang, D.N. Finegold, J.P. Landers, *Anal. Chem.* 72 (2000) 2765.
- [36] J.C. Fanguy, C.S. Henry, *Electrophoresis* 23 (2002) 767.
- [37] J. Wang, M.P. Chatrathi, *Anal. Chem.* 75 (2003) 525.
- [38] C.D. Garcia, C.S. Henry, *Analyst* 129 (2004) 579.
- [39] J. Wang, M.P. Chatrathi, G.E. Collins, *Anal. Chim. Acta* 585 (2007) 11.
- [40] Y. Du, J.L. Yan, W.Z. Zhou, X.Y. Yang, E.K. Wang, *Electrophoresis* 25 (2004) 3853.
- [41] V. Srinivasan, V.K. Pamula, R.B. Fair, *Lab Chip* 4 (2004) 310.
- [42] H. Nakamura, Y. Murakami, E. Tamiya, I. Karube, *Anal. Chem.* 73 (2001) 373.
- [43] H. Moon, A.R. Wheeler, R.L. Garrell, J.A. Loo, C.J. Kim, *Lab Chip* 6 (2006) 1213.
- [44] P. Caglar, S.A. Tuncel, N. Malcik, J.P. Landers, J.P. Ferrance, *Anal. Bioanal. Chem.* 386 (2006) 1303.
- [45] T. Noda, H. Takao, K. Yoshioka, N. Oku, M. Ashiki, K. Sawada, K. Matsumoto, M. Ishida, *Sens. Actuators B* 119 (2006) 245.
- [46] W.S.B. Yeung, G.A. Luo, Q.G. Wang, J.P. Ou, *J. Chromatogr. B* 797 (2003) 217.
- [47] A.E. Herr, D.J. Throckmorton, A.A. Davenport, A.K. Singh, *Anal. Chem.* 77 (2005) 585.
- [48] A.E. Herr, A.V. Hatch, D.J. Throckmorton, H.M. Tran, J.S. Brennan, W.V. Giannobile, A.K. Singh, *PNAS* 104 (2007) 5268.
- [49] T.M. Phillips, *Electrophoresis* 25 (2004) 1652.
- [50] T.M. Phillips, E. Wellner, *J. Chromatogr. A* 1111 (2006) 106.
- [51] M.G. Roper, J.G. Shackman, G.M. Kahlgren, R.T. Kennedy, *Anal. Chem.* 75 (2003) 4711.
- [52] T. Lim, H. Ohta, T. Matsunaga, *Anal. Chem.* 75 (2003) 3316.
- [53] K. Sato, M. Tokeshi, H. Kimura, T. Kitamori, *Anal. Chem.* 73 (2001) 213.
- [54] K. Tsukagoshi, N. Jinno, R. Nakajima, *Anal. Chem.* 77 (2005) 1684.
- [55] J. Moorthy, G.A. Mensing, D. Kim, S. Mohanty, D.T. Eddington, W.H. Tepp, E.A. Johnson, D.J. Beebe, *Electrophoresis* 25 (2004) 1705.
- [56] V. Linder, E. Verpoorte, N.F. De Rooij, H. Sigris, W. Thormann, *Electrophoresis* 23 (2002) 740.
- [57] M. Wolf, D. Juncker, B. Michel, P. Hunziker, E. Delamarche, *Biosens. Bioelectron.* 19 (2004) 1193.
- [58] R. Kurita, Y. Yokota, Y. Sato, F. Mizutani, O. Niwa, *Anal. Chem.* 78 (2006) 5525.
- [59] G. Chen, Y.H. Lin, J. Wang, *Talanta* 68 (2006) 497.
- [60] L. Marle, G.M. Greenway, *Trends Anal. Chem.* 24 (2005) 795.
- [61] J. Wang, J. Zima, N.S. Lawrence, M.P. Chatrathi, *Anal. Chem.* 76 (2004) 4721.
- [62] J. Wang, M.P. Chatrathi, *Anal. Chem.* 73 (2001) 1804.
- [63] J. Wang, M. Pumera, G.E. Collins, A. Mulchandani, *Anal. Chem.* 74 (2002) 6121.
- [64] A. Hilmi, J.T. Luong, *Environ. Sci. Technol.* 34 (2000) 3046.
- [65] J. Fischer, J. Barek, J. Wang, *Electroanalysis* 18 (2006) 195.
- [66] D. Shin, D.A. Tryk, A. Fujishima, A. Muck Jr., G. Chen, J. Wang, *Electrophoresis* 25 (2004) 3017.
- [67] J. Wang, M.P. Chatrathi, B. Tian, *Anal. Chim. Acta* 416 (2000) 9.
- [68] C.D. Garcia, G. Engling, P. Herckes, J.L. Collett Jr., C. Henry, *Environ. Sci. Technol.* 39 (2005) 618.
- [69] Y. Ding, C.D. Garcia, *Analyst* 131 (2006) 208.
- [70] S. Wakida, A. Chiba, T. Matsuda, K. Fukushi, H. Nakanishi, X. Wu, H. Nagai, S. Kurosawa, S. Takeda, *Electrophoresis* 22 (2001) 3505.
- [71] I. Chang, J.J. Tulock, J. Liu, W. Kim, D.M. Cannon Jr., Y. Lu, P.W. Bohn, J.V. Sweedler, D.M. Crokek, *Environ. Sci. Technol.* 39 (2005) 3756.
- [72] Y. Xu, F.G. Bessoth, J.C.T. Eijkel, A. Manz, *Analyst* 125 (2000) 677.
- [73] S. Fujii, T. Tokuyama, M. Abo, A. Okubo, *Anal. Sci.* 20 (2004) 209.
- [74] G. Deng, G.E. Collins, *J. Chromatogr. A* 989 (2003) 311.
- [75] R. Bodor, V. Madajova, D. Kaniansky, M. Masar, M. Johnck, B. Stanislawski, *J. Chromatogr. A* 916 (2001) 155.
- [76] A. Escarpa, M.C. Gonzalez, A.G. Crevillén, A.J. Blasco, *Electrophoresis* 28 (2007) 1002.
- [77] M. Scampicchio, J. Wang, S. Mannino, M.P. Chatrathi, *J. Chromatogr. A* 1049 (2004) 189.
- [78] P. Kuban, P.C. Hauser, *Electrophoresis* 26 (2005) 3169.
- [79] W.S. Law, P. Kuban, J.H. Zhao, F.Y. Li, P.C. Hauser, *Electrophoresis* 26 (2005) 4648.
- [80] Q. Chu, Y. Guan, C. Geng, J. Ye, *Anal. Lett.* 39 (2006) 729.
- [81] R.W. Hompesch, C.D. Garcia, D.J. Weiss, J.M. Vivanco, C.S. Henry, *Analyst* 5 (2005) 694.
- [82] A.J. Blasco, I. Barrigas, M.C. Gonzalez, A. Escarpa, *Electrophoresis* 26 (2005) 4664.
- [83] A.G. Crevillén, A.J. Blasco, M.C. Gonzalez, A. Escarpa, *Electrophoresis* 27 (2006) 5110.
- [84] M. Avila, M.C. Gonzalez, M. Zougagh, A. Escarpa, A. Rios, *Electrophoresis* 28 (2007) 4233.
- [85] M. Kato, Y. Gyoten, K. Sakai-Kato, T. Toyo'oka, *J. Chromatogr. A* 1013 (2003) 183.

- [86] A.J. Blasco, A.G. Crevillén, P. de la Fuente, M.C. Gonzalez, A. Escarpa, *Analyst* 132 (2007) 323.
- [87] D. He, Z. Zhang, Y. Huang, Y. Hu, *Food Chem.* 101 (2007) 667.
- [88] Y. Dong, K.S. Phillips, Q. Cheng, *Lab Chip* 6 (2006) 675.
- [89] S. Han, J.H. Chob, I.H. Choa, E.H. Paek, H.B. Ohc, B.S. Kim, C. Ryu, K. Lee, Y.K. Kim, S.H. Paek, *Anal. Chim. Acta* 587 (2007) 1.
- [90] D. Hoegger, P. Morier, C. Vollet, D. Heini, F. Reymond, J.S. Rossier, *Anal. Bioanal. Chem.* 387 (2007) 267.

Review

Nanomaterial-based electrochemical DNA sensing strategies

Arzum Erdem*

Ege University, Faculty of Pharmacy, Analytical Chemistry Department, 35100 Bornova, Izmir, Turkey

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In honor of Prof. Joseph Wang's 60th birthday who is the pioneer of electrochemical (bio)sensors.

Abstract

DNA sensing strategies have recently been varied with the number of attempts at the development of different biosensor devices based on nanomaterials, which will further become DNA microchip systems. The investigations at the side of material science in connection with electrochemical biosensors open new directions for detection of specific gene sequences, and nucleic acid–ligand interactions.

An overview is reported here about nanomaterial-based electrochemical DNA sensing strategies principally performed for the analysis of specific DNA sequences and the quantification of nucleic acids. Important features of electrochemical DNA sensing strategies, along with new developments based on nanomaterials are described and discussed.

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Keywords: Nanomaterials; Biosensors; DNA; Electrochemical transducers; Nanoparticles; Carbon nanotubes; Guanine; Adenine

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1. Introduction

Recent progress in biosensing technologies based on nanomaterials has resulted by the development of several novel sensor devices with their challenging applications. Modern biomedical sensors developed with advanced microfabrication and signal processing approaches are becoming inexpensive, accurate, and reliable. This progress in miniature devices and instrumentation development will significantly impact the practice of medical

care as well as future advances in the biomedical industry [1]. Electrochemical, optical, and acoustic wave sensing technologies have currently emerged as some of the most promising biosensor technologies.

The use of nucleic acid technologies has significantly improved preparation and diagnostic procedures in life sciences. Various combination of DNA associated with different types of transducers are an attractive subject of research. Nucleic acid layers combined with electrochemical or optical transducers produce a new kind of affinity biosensors as DNA biosensor for small molecular weight molecules [1–6]. The detection of DNA has a particular interest in genetics, pathology, criminology, pharmacogenetics, food safety and many other fields.

* Tel.: +90 232 388 0110x5131; fax: +90 232 388 5258.E-mail address: arzum.erdem@ege.edu.tr.

After discovery of electroactivity in nucleic acids at the beginning of the 1960s [7], many approaches in combination with electrochemical nucleic acid sensors have been developed for analyzing or quantification of nucleic acids and DNA interactions and recognition events in solution and at solid substrates [1–3,8–31]. Electrochemical DNA biosensors are attractive devices especially for converting DNA hybridization event into an analytical signal for obtaining sequence-specific information in connection with clinical, environmental or forensic investigations. Such fast on-site monitoring schemes are required for quick preventive action and early diagnosis.

Nucleic acid hybridization is a process in which inconsonant nucleic acid strands with specific organization of nucleotide bases exhibiting complementary pairing with each other under specific given reaction conditions, thus forms a stable duplex molecule. This phenomenon is possible because of the biochemical property of base pairing, which allows fragments of known sequences to find complementary matching sequences in an unknown DNA sample [6]. An increasing interest has appeared in the development of simple, rapid and user-friendly electrochemical detection systems based on DNA sequence and mutant gene analysis, for instance early and precise diagnosis of infectious agents, for routine clinical tests [8,10–17,23,29]. Thus, DNA hybridization biosensors can be employed for determining early diagnoses of infectious agents in various environments [1,2] and these devices can be exploited for monitoring sequence-specific hybridization events directly [9,13–17] based on the oxidation signal of guanine/adenine or using DNA intercalators (some antibiotics, metal coordination complexes, etc.) which contain several aromatic condensed rings and often bind dsDNA in an intercalative mode [8,18,19,21,23,27,29,30].

Material science has recently a growing interest since it can present the possibilities how to apply novel materials from micro- to nanoscales, such as nanoparticles, nanotubes, nanowires into optical, electrical, magnetic, chemical and biological applications [32–44]. The novel surfaces modified with nanomaterials have recently presented an excellent prospect for biological recognition surfaces in order to develop a more selective and sensitive DNA sensor technology.

In the following section, the important features of electrochemical DNA sensing strategies, along with new developments based on nanomaterials are described and discussed.

2. Nanomaterial-based electrochemical DNA sensing strategies

Progress in synthesis and characterization of nanostructured materials and continuously emerging nanotechnologies promise dramatic changes in sensor design and their capabilities. Various nanostructured and advanced electronic materials with remarkable electrical, optical, and mechanical properties have recently been developed, with numerous unique applications [45].

Electrochemical DNA biosensors can normally be employed for determining the possible interaction between drug and DNA, or early and precise diagnoses of infectious agents in various environments [1–5] by using different electrochemical tech-

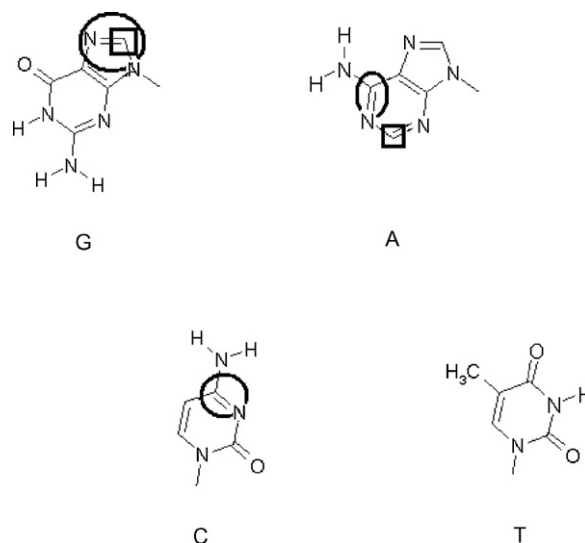
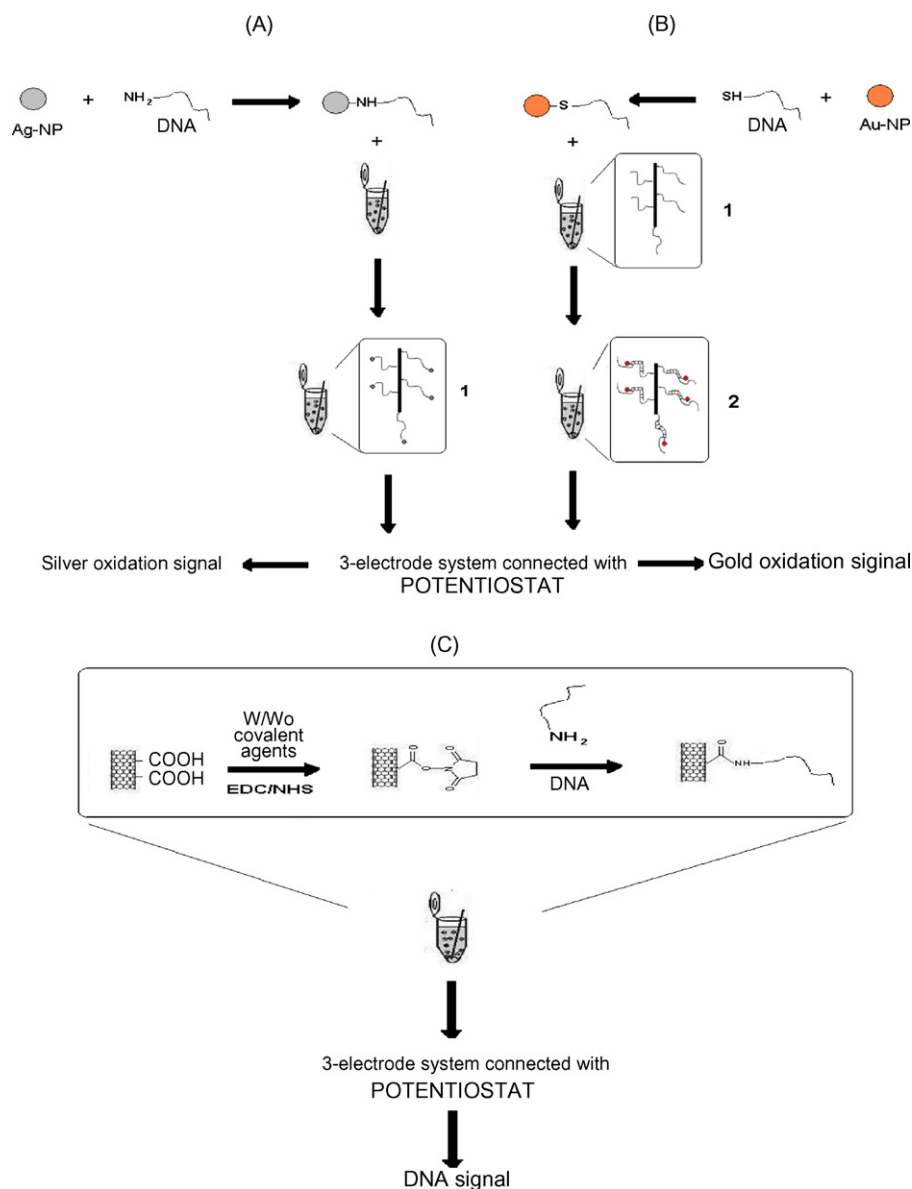


Fig. 1. All purine and pyrimidine bases of DNA and their electroactive sites; the circles representing the reducible (Red) groups and the squares representing the oxidizable (Oxi) groups.

niques; differential pulse voltammetry (DPV), potentiometric stripping analysis (PSA), square wave voltammetry (SWV), cathodic stripping voltammetry (CSV), adsorptive transfer stripping voltammetry (AdSTV), linear voltammetry (LV) and linear square voltammetry (LSV), etc. The reported studies utilized in DNA sensing strategies combined with different electrochemical transducers; carbon paste electrode (CPE)/magneto carbon paste electrode (MCPE), hanging mercury drop electrode (HMDE), screen printed electrode (SPE), pencil graphite electrode (PGE), pyrolytic graphite electrode (PrGE), mercury film electrode (MFE), gold electrode (AuE), platinum electrode (PtE), include:

- (1) label-free DNA detection system called for sequence-specific hybridization processes based on the redox signal of most electroactive DNA bases, guanine and adenine [9,13–18,34,35,37,40,41] (all purine and pyrimidine bases of DNA, and their electroactive sites have also been shown in Fig. 1);
- (2) electroactive indicator-based system (a) in the presence of any DNA intercalators (metal coordination complexes, antibiotics, etc.) [8,19,23,27,30], and (b) in the presence of some metal tags labelled nanoparticles, such as gold and silver nanoparticles, etc. [32,36,38,39,43,44].

In recent years, different electrochemical DNA sensing strategies developed in principle of nanotechnology have become one of the most exciting forefronts fields in analytical chemistry due to the challenging advances of various nanomaterials, e.g., magnetic particles/nanoparticles labelled with metal tags [14–17,36–39,41–44,46,47], nanotubes [34,35,40,48,49] and nanowires [33,50–52] by using different electrochemical transducers. Especially, after the pencil lead electrode (PGE) was introduced by Wang et al. [53] under the principles of development for a single-use nucleic acid sensor technology, the



Scheme 1. Representative schemes for presenting the simple applications of nanomaterial-based electrochemical strategies performed using disposable graphite electrodes, PGE. (A) Silver nanoparticles (Ag-NPs) and amino-linked DNA are used. Step 1 represents the immobilization of Ag-NPs labelled DNA onto the surface of PGE. (B) Gold nanoparticles (Au-NPs) and thiol-linked DNA are used. Step 1 represents the immobilization of DNA probe onto the surface of PGE and step 2 represents the hybridization between probe and its complementary labelled with Au-NPs. (C) Carbon nanotubes (CNTs), covalent agents (EDC/NHS) and amino-linked DNA are used.

numerous electrochemical DNA sensing routes have been created and then, progressed using disposable graphite electrodes. In comparison to the strategies performed using other electrochemical transducers, AuE, GCE, CPE and HMDE, etc. the applications of different nanomaterials-based electrochemical DNA sensing strategies using disposable graphite electrodes, PGE (representative simple procedures shown in Scheme 1) have been found simpler and faster. For example, to develop electrochemical DNA sensing approaches using AuE, GCE or GEC electrodes, the time-consuming cleaning procedure and complicated surface chemistry process are required for the preparation of these electrodes. Consequently, these strategies based on various nanomaterials in combination with PGEs bring

some important advantages such as being inexpensive, simple and direct electrochemical assay for DNA detection in more reproducible and more sensitive results with a good degree of selectivity.

2.1. Electrochemical DNA sensing strategies using magnetic particles/nanoparticles connected with biological molecules labelled with metal tags

The use of magnetic particles/nanoparticles labelled with metal tags can bring novel capabilities to bioaffinity assays and sensors, especially after the electrochemical DNA detection strategies on nanoparticles have recently been introduced.

Table 1

A summary of the recent electrochemical investigations for DNA detection strategies based on various type of particles

Technique	Working electrode	HT (min)	Response	DL	Reference
PSA	PGE, CPE, m-CPE	10	Guanine	60 pM	[14]
DPV	GECE, m-GECE	20	Guanine	9.68 fmol/mL	[15]
DPV	PGE	20	Guanine	74.8 fmol/mL	[17]
PSA	SPEs	30	Silver	1.2 fmol	[37]
PSA	MFE	10	Cadmium	100 fmol in 50 μ L sample	[38]
CSV	HMDE	15	Iron	10 ng in 50 μ L sample	[39]
DPV	PGE	20	Guanine	43.11 pmol/mL	[41]
DPV, SWV, LV	SPEs	20	α -Naphthol	500 pg in 50 μ L sample	[42]
DPV	CPE, PGE	60	Gold	0.78–0.83 fmol/mL	[43]
	m-GECE	15		33 pmol	[62]
AdTS-SWV	PrGE	30	Guanine/adenine	Higher than ppb level	[47]
LSV			1-Naphthol	3 fmol	
DPV	PGE	–	Silver	–	[44]
CSV	HMDE	30	Adenine	Below 2 nM for adenine	[54]
PSA	CPE, MCPE	–	Guanine	–	[55]

Working electrodes: carbon paste electrode (CPE)/magneto carbon paste electrode (m-CPE), hanging mercury drop electrode (HMDE), graphite epoxy composite electrode (GECE)/magneto graphite epoxy composite electrode (m-GECE), screen printed electrode (SPE), pencil graphite electrode (PGE), pyrolytic graphite electrode (PrGE) and mercury film electrode (MFE). Voltammetric techniques: differential pulse voltammetry (DPV), potentiometric stripping analysis (PSA), square wave voltammetry (SWV), cathodic stripping voltammetry (CSV), adsorptive transfer stripping voltammetry (AdSTV), linear voltammetry (LV) and linear square voltammetry (LSV). DL: detection limit, HT: hybridization time and Ref: related reference.

In the majority of earlier reports (also summarized in Table 1) it was shown that different types of transducers in connection with a number of voltammetric techniques were used for the development of efficient tools on electrochemical DNA sensing technology in combination with various type of particles. Such protocols have been developed by using the colloidal gold tags, semiconductor quantum dot tracers, polymeric carrier beads, or magnetic particles (summarized in Fig. 2).

In table, an overview about DNA sensing strategies by using magnetic particles and nanoparticles labelled with metals is briefly summarized, and their applications for the development of electrochemical sensor technology are discussed.

The electrochemical DNA detection using magnetic particles [14–17,41,42,46,47,55] brings the sequence-specific detection of DNA hybridization observed in exceedingly low detection limits as resulting in efficient magnetic separation. For example, Wang et al. [14] was reported a novel genomagnetic electrochemical assay related to BRCA1 breast-cancer gene based on label-free detection by using different transducers, PGE, CPE, and also m-CPE. An enzyme-linked sandwich hybridization was also studied combined with electrochemical detection of DNA sequences related to BRCA1 gene by using magnetic particles labelled with probe hybridizing to a biotinylated DNA target capturing a streptavidin-alkaline phosphatase (AP) enzyme, and consequently, 1-naphthol was measured as a product of enzymatic reaction in the presence of DNA hybridization [42]. Another study on enzyme-linked immunoassay coupling with magnetic particles for the detection of the DNA hybridization by using linear square voltammetry (LSV) technique and pyrolytic graphite electrode (PrGE) was reported by Palecek et al. [47]. Recently, there have been two reports performed by Erdem et al. [15,17] representing the electrochemical detection routes for DNA hybridization related to specific sequences using

different transducers. A label-free genomagnetic assay for the electrochemical detection of *Salmonella* spp. sequence has been presented by using graphite-epoxy composite electrode (GECE) and magneto-GEC electrodes as electrochemical transducers [15]. Another genomagnetic assay developed by Erdem et al. [17] by using commercial magnetic particles for the electrochemical monitoring of detection of wild type hepatitis B virus (HBV) DNA in polymerase chain reaction (PCR) amplicons in length 437-bp has been described.

In contrast to other similar methodologies earlier reported in the literatures, as the first time, the streptavidin-coated magnetic nanoparticles were produced in the average diameter of 125 and 225 nm, and their performance was studied for the development of electrochemical DNA sensor technology [41]. Thus, it was exhibited that DNA hybridization can be realized onto magnetic nanoparticles carrying the probe oligonucleotides with the target sequences within the medium, and it can be effectively followed by the measurement of guanine oxidation signal using an electrochemical nucleic acid sensor in order to detect specific DNA sequences related to hepatitis B virus (HBV) quite sensitively and selectively, with this less time-consuming, and cheaper label-free electrochemical technique as the first time using home-made magnetic nanoparticles by Erdem et al. [41] in comparison to other traditional techniques [29,56,57] reported in literatures, where several external indicators $[\text{Co}(\text{phen})_3]^{3+}$, di(2,2'-bipyridine)osmium(III) complexes, methylene blue, etc. have been used.

Recent developments have led to the progress of functional nanoparticles that could bind to nucleic acids, peptides, and proteins by applying the principles of surface chemistry. The electrochemical signal coming from nanoparticles labelled with gold (Au) tags were mostly used for the development of many strategies on electrochemical DNA detection

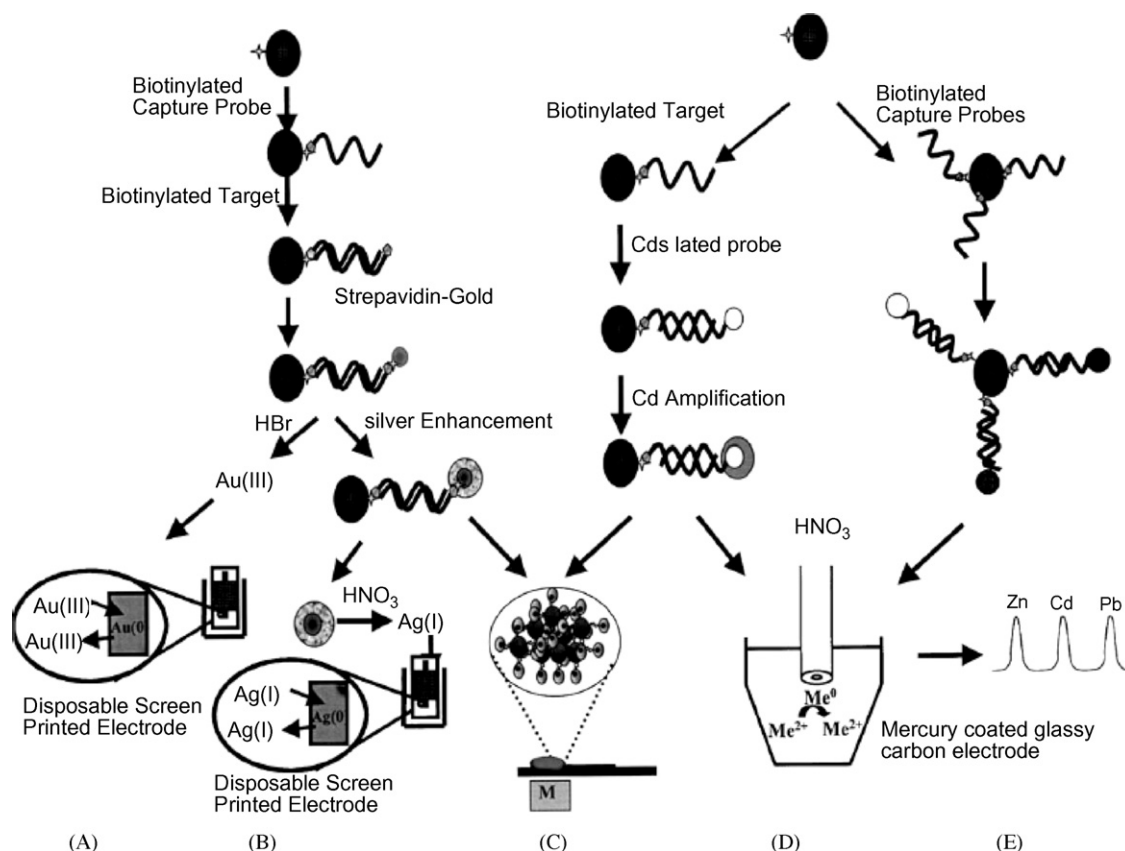


Fig. 2. Particle-based protocols for electrochemical detection of DNA. These assays involve the introduction of the probe-attached onto the magnetic particles, addition of the target/hybridization event, magnetic removal of unwanted materials, binding of the metal and amplified electrochemical detection of the dissolved gold (Au) (A), silver (Ag) (B) and cadmium sulfide (CdS) (D) nanoparticles. Me: metal tag. Also shown are solid-state stripping (C) and multi-target (E) detection protocols. (Reprinted from [5], Copyright (2003) with permission from Elsevier.)

[43,46,58,59]. The electrochemical detection and amplification of DNA hybridization based on streptavidin-coated Au nanoparticles was reported as the first time by Wang et al. [58]. The acid dissolution of Au tags was monitored by chronopotentiometric stripping analysis at disposable electrodes. Authier et al. [46] presented a method using Au labelled probes for the detection of human cytomegalovirus in PCR amplicons. After the release of gold atoms by oxidative metal dissolution using acidic bromine–bromide solution, the signal of gold was measured by anodic stripping voltammetry (ASV).

A sensitive electrochemical detection assay for DNA hybridization using silver nanoparticles and ASV method connected with carbon fiber ultramicroelectrode was reported by Cai et al. [60]. In this study, the determination of solubilized Ag(I) ions was successfully performed after the release of silver atoms by oxidative metal dissolution. Zhu et al. [61] reported a method for the detection of DNA hybridization in connection to lead sulfide (PbS) nanoparticles by measuring the lead signal in combination with ASV technique and polymer-modified glassy carbon electrode.

A novel nanoparticle-based protocol for detecting DNA hybridization was performed using a strategy based on a magnetically induced solid-state electrochemical stripping detection of silver in connection with single-use electrodes [37]. The

selectivity of this assay was also checked in co-existing of a number of mismatched oligonucleotides and noncomplementary oligonucleotides beside the complementary of probe. Another strategy for the detection of DNA hybridization in a higher sensitivity with the shortest time (i.e., 10 min hybridization time) followed by the genomagnetic assay – magnetic-bead/ DNA hybrid/ cadmium sulfide nanoparticle – was performed successfully using mercury-film electrode [38]. Two different particle-based electrochemical schemes were reported for monitoring DNA hybridization based on PSA detection of an iron tracer [39]. The probes labelled with gold-coated iron core-shell nanoparticles were used, and thus, the captured iron-containing particles are dissolved following hybridization step, the released iron is quantified by cathodic-stripping voltammetry by using HMDE, in the presence of the 1-nitroso-2 naphthol ligand and a bromate catalyst. The results showed that this approach offers a novel DNA sensing strategy in a high sensitivity with minimal contributions from noncomplementary nucleic acids.

In the one of recent studies [43], the electroactivity of Au nanoparticles was used for the detection of hybridization without using any external indicators, or the need for any acidic dissolution of Au tag. Thus, DNA-specific sequences related to factor V Leiden mutation were detected electrochemically in this study by tagging a probe with gold colloid, and immobilizing the tar-

get onto the disposable electrode followed by anodic stripping analysis of Au colloid in a higher sensitivity and selectivity. The work also has a realistic potential application, since the experiments were carried out using real PCR amplicons. Pumera et al. described two gold nanoparticles-based genomagnetic sensors for detection of DNA hybridization related to specific DNA sequences, i.e., BRCA1 and cystic fibrosis. Consequently, the direct electrochemical detection of gold tags in the presence of hybridization was performed successfully by using magnetic graphite-epoxy composite electrodes (m-GECE). In contrast to the detection limit reported in the study of Pumera and coworkers [62], the lower detection limit as fM concentration level was obtained in this study [43] with a higher hybridization time (i.e., 60 min) and using PCR amplicons.

Some literatures have shown that the quantum dots (QD) can be used in a variety of bioanalytical formats with electrochemical detection, especially for DNA [63]. In this study, a novel gold nanoparticle-based protocol for detection of DNA hybridization based on a magnetically triggered direct electrochemical detection of gold quantum dot tracers by using m-GECE was described. Au67 quantum dot tag in the size of 1.4 nm linked to the target DNA was directly detected after the DNA hybridization event, without need of any acidic dissolution.

A novel electrochemical assay for the improved electrochemical sensing of DNA based on both oxidation signals of silver (without any external catalyst for metal ion or any acidic dissolution) and also guanine by using disposable pencil graphite electrodes (PGE) was introduced to the literatures [44]. The easy surface modification of disposable electrodes with nucleic acids was performed in this study by passive adsorption using amino-linked DNA oligonucleotide attached onto the surface of silver nanoparticles (Ag-NPs). This electrochemical approach for DNA detection has presented some important advantages in comparison to other earlier studies [37–39,58–61] such as, low preparation cost and easy modification of surface materials in higher sensitivity and selectivity.

2.2. Electrochemical DNA sensing strategies using carbon nanotubes and other nanomaterials

The versatility of the carbon–carbon bond presents the opportunity for attaching different functional groups to the end of the carbon nanotube (CNT) that offers potential for CNTs to be used as a new material for sensors in (bio)chemical applications [64].

The modification of electrochemical transducers with carbon nanotubes (CNTs) has recently attracted considerable attention in the field of DNA sensing technology and thus, many different schemes for electrochemical DNA sensing based on CNTs have been presented in the literatures [34,35,40,48,49,64].

Direct electrochemistry of DNA electroactive bases, guanine and adenine at a multiwalled carbon nanotube (MWNT)-modified glassy carbon electrode (GCE) provided significantly enhanced voltammetric signals (with calculated detection limit as 100 fmol of breast cancer BRCA1 gene) in comparison to unmodified GCE by Wang et al. [34].

The fabrication of CNT riched paste electrode was fabricated by Pedano et al. [35], and it was used for adsorption

and electrochemical oxidation of nucleic acids. Incorporation of multiwalled nanotubes (MWNT) into carbon paste matrix provided 29 and 61 fold larger current values than the ones obtained from a carbon paste electrode for single stranded DNA (ssDNA) and short oligonucleotide. Additionally, the use of CNTs was reported for enzyme amplification of electrochemical DNA sensing strategy by Wang et al. [48].

A nanoelectrode array based on vertically aligned multiwalled carbon nanotubes, MWCNTs with controlled density, embedded in a SiO₂ matrix was reported by Li's group to be useful for detecting DNA hybridization [49]. Oligonucleotide probes were selectively functionalised to the open ends of the MWNTs and thus, DNA targets could be detected by combining the nanoelectrode array with ruthenium bipyridine mediated guanine oxidation.

A simple and sensitive electrochemical method based on CNT-modified disposable graphite electrodes for the detection of DNA and label-free DNA hybridization was performed by using the signal enhancement of the guanine oxidation signal without any modifications in the native bases or any external labelling by Erdem et al. [40]. Both CNT-modified transducers displayed an attractive voltammetric performance over their bare ones, the modified PGE compared favorably to the commonly used CNT-modified GCE electrode.

The specific properties of other nanomaterials, such as nanowires also offer an excellent prospect for biological recognition surfaces in order to develop a more selective and sensitive biosensor technology [33,50–52]. Li et al. [51] reported a novel method using a sequence-specific label-free DNA sensors based on silicon nanowires (Si-NWs) by measuring the change of the conductance. Kelley and coworkers [52] developed a gold nanowire array (Au-NW) in 15–20 nm in diameter, and this array was used for electrochemical DNA detection by the help of the electrocatalytic reporter systems, Ru(NH₃)₆³⁺ and Fe(CN)₆³⁻.

3. Conclusions and future perspectives

Nanotechnology refers to research and technology development at the atomic, molecular, and macromolecular scale, leading to the controlled manipulation and study of structures and devices with length scales from 1 to 100 nm range [65]. Nanomaterials have unique chemical and physical properties that offer important possibilities for analytical chemistry. For example, nanoparticles represent an excellent biocompatibility with biomolecules, and display unique structural, electronic, magnetic, optical and catalytic properties which have made them a very attractive material [66] as labels in the detection of DNA hybridization [67] using optical methods, e.g., surface plasmon resonance [68] or different electrochemical techniques [5] between other applications.

The integration of nanotechnology in combination with molecular biology and electrochemistry has been expected to create major advances in the area of electrochemical DNA sensor technology. The development of advanced electrochemical DNA sensing strategies based on nanomaterials have recently been considered as important tools in the field of genomics, medical diagnosis, and drug–DNA interactions [36,50,64].

The electrochemical schemes for DNA detection based on magnetic particles assay in combination with metal nanoparticles or enzyme labelling, or using label-free system, brings the sequence-specific detection of DNA hybridization observed in exceedingly low detection limits as resulting in efficient magnetic separation.

Such coupling of DNA hybridization surfaces with electrochemical transducers and metal nanoparticles eliminates the needs for external indicators and advanced surface modification or other regeneration schemes.

The modification of transducers with carbon nanotubes has recently attracted considerable attention in the field of electro-analytical chemistry. The high-surface area, and hollow geometry, the useful mechanical properties of CNTs combined with their electronic conductivity and ability to promote electron transfer reactions provide novel challenging transducers for the catalysis of biomolecules and inorganic compounds [69].

The exploitation of carbon nanotubes for the development of electrochemical DNA sensing strategies has been still in progress. Beside this progress on the development of nanotube-based electrochemical transducers, there have been available reports in the literatures that represent the results obtained by (1) any external time-consuming step required expensive agents (e.g., enzyme labelling of CNT for specific binding of DNA onto the surface), or (2) any fluorescence labels for detection of DNA, and DNA hybridization.

The development of DNA sensing strategies or gene detection has been increasing its practical importance, especially in conjunction with the development of microfabrication technology toward chips and arrays. It is hoped that continued development through combined efforts in microelectronics, surface/interface chemistry, molecular biology, and analytical chemistry will lead to the establishment of genosensor technology based on DNA sensing strategies combined with the advantages of nanotechnology.

Nanomaterial-based genelectronics, the molecular interfacing approach into exploiting DNA recognition events is important in coming perspective, that can bring us the term as “DNA microarray” to measure the expression patterns of thousands of genes in parallel, generating clues to gene function that can help to identify appropriate targets for therapeutic intervention, and to monitor changes in gene expression in response to drug treatments [70–72].

Congratulations

I feel very, very lucky to have been able to work with Prof. Wang at his senso-chip lab. His outstanding example of scientific excellence allows us always to work in a successful and challenging atmosphere. In addition, his happy and friendly personality encourages us to join with the scientific community in becoming a good and close friend.

I would like to congratulate Prof. Wang on his 60th birthday and I wish him with his family more and more wonderful years filled with health, happiness and continued success in all of his endeavors!



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References

- [1] J. Wang, *Nucleic Acids Res.* 28 (2000) 3011–3016.
- [2] E. Palecek, M. Fojta, *Anal. Chem.* 73 (2001) 75A–83A.
- [3] A. Erdem, M. Ozsoz, *Electroanalysis* 14 (2002) 965–974.
- [4] M.I. Pividori, A. Merkoci, S. Alegret, *Biosens. Bioelectron.* 15 (2000) 291–303.
- [5] J. Wang, *Anal. Chim. Acta* 500 (2003) 247–257.
- [6] A.K. Bej, *Nucleic Acid Analysis: Principles and Bioapplications*, Wiley-Liss Press, New York, 1996, pp. 1–29 (Chapter 1).
- [7] E. Palecek, *Nature* 188 (1960) 656–657.
- [8] K. Millan, A. Saraulo, S.R. Mikkelsen, *Anal. Chem.* 66 (1994) 2943–2948.
- [9] J. Wang, G. Rivas, J.R. Fernandes, J.L. Lopez Paz, M. Jiang, R. Waymire, *Anal. Chim. Acta* 375 (1998) 197–203.
- [10] E. Palecek, M. Fojta, M. Tomschik, J. Wang, *Biosens. Bioelectron.* 13 (1998) 621–628.
- [11] J. Wang, G. Rivas, D. Luo, X. Cai, F.S. Valera, N. Dontha, *Anal. Chem.* 68 (1996) 4365–4369.
- [12] J. Wang, G. Rivas, D. Luo, X. Cai, F.S. Valera, N. Dontha, P.A.M. Farias, H. Shiraishi, *Anal. Chem.* 68 (1996) 2251–2254.
- [13] A. Erdem, M.I. Pividori, M. Del Valle, S. Alegret, *J. Electroanal. Chem.* 567 (2004) 29–37.
- [14] J. Wang, A.-N. Kawde, A. Erdem, M. Salazar, *Analyst* 126 (2001) 2020–2024.
- [15] A. Erdem, M.I. Pividori, A. Lermo, A. Bonanni, M. Del Valle, S. Alegret, *Sens. Actuator B: Chem.* 114 (2006) 591–598.
- [16] J. Wang, G.-U. Flechsig, A. Erdem, O. Korbut, P. Gründler, *Electroanalysis* 16 (11) (2004) 928–931.
- [17] A. Erdem, D. Ozkan Ariksoysal, H. Karadeniz, P. Kara, A. Sengonul, A.A. Sayiner, M. Ozsoz, *Electrochem. Commun.* 7 (8) (2005) 815–820.
- [18] E. Palecek, M. Tomschik, V. Stankova, L. Havran, *Electroanalysis* 9 (1997) 990–997.
- [19] A. Erdem, M. Ozsoz, *Anal. Chim. Acta* 437 (2001) 107–114.
- [20] A. Erdem, M. Ozsoz, *Turk. J. Chem.* 25 (2001) 469–475.
- [21] J. Wang, G. Rivas, X. Cai, H. Shiraishi, P.A.M. Farias, N. Dontha, D. Luo, *Anal. Chim. Acta* 332 (1996) 139–144.
- [22] F. Jelen, A. Erdem, E. Palecek, *Bioelectrochemistry* 55 (2002) 165–167.
- [23] G. Marrazza, G. Chiti, M. Mascini, M. Anichini, *Clin. Chem.* 46 (2000) 31–37.
- [24] M. Ozsoz, A. Erdem, P. Kara, K. Kerman, D. Ozkan, *Electroanalysis* 15 (7) (2003) 613–619.
- [25] H. Karadeniz, B. Gulmez, F. Sahinci, A. Erdem, G.I. Kaya, N. Unver, B. Kivcak, M. Ozsoz, *J. Pharm. Biomed. Anal.* 33 (2003) 295–302.
- [26] A. Erdem, B. Kosmider, E. Zyner, R. Osiecka, J. Ochocki, M. Ozsoz, *J. Pharm. Med. Anal.* 38 (4) (2005) 645–652.
- [27] S.O. Kelley, E.M. Boon, J.K. Barton, N.M. Jackson, M.G. Hill, *Nucleic Acid Res.* 27 (1999) 4830–4837.

- [28] A.M.O. Brett, T.R.A. Macedo, D. Raimundo, M.H. Marques, S.H.P. Serrano, *Biosens. Bioelectron.* 13 (1998) 861–867.
- [29] A. Erdem, K. Kerman, B. Meric, U.S. Akarca, M. Ozsoz, *Anal. Chim. Acta* 422 (2000) 139–149.
- [30] H. Karadeniz, A. Erdem, B. Gulmez, F. Jelen, M. Ozsoz, E. Palecek, *Front. Biosci.* 11 (2006) 1870–1877.
- [31] M. Fojta, R. Doffkova, E. Palecek, *Electroanalysis* 8 (1996) 420–426.
- [32] A.D. Mc Farland, R.P. Van Duyne, *Nano Lett.* 3 (2003) 1057–1062.
- [33] A.K. Salem, J. Chao, K.W. Leong, P.C. Searson, *Adv. Mater.* 16 (2004) 268–271.
- [34] J. Wang, A.-N. Kawde, M. Musameh, *Analyst* 128 (2003) 912–916.
- [35] M.L. Pedano, G.A. Rivas, M.L. Pedano, *Electrochem. Commun.* 6 (2004) 10–16.
- [36] J. Wang, *Analyst* 130 (2005) 421–426.
- [37] J. Wang, D. Xu, R. Polsky, *JACS* 124 (2002) 4208–4209.
- [38] J. Wang, L. Guodong, R. Polsky, A. Merkoci, *Electrochem. Commun.* 4 (2002) 722–726.
- [39] J. Wang, L. Guodong, A. Merkoci, *Anal. Chim. Acta* 482 (2003) 149–155.
- [40] A. Erdem, P. Papakonstantinou, H. Murphy, *Anal. Chem.* 78 (2006) 6656–6659.
- [41] A. Erdem, F. Sayar, H. Karadeniz, G. Guven, M. Ozsoz, E. Piskin, *Electroanalysis* 19 (2007) 798–804.
- [42] J. Wang, D. Xu, A. Erdem, R. Polsky, M. Salazar, *Talanta* 56 (2002) 931–938.
- [43] M. Ozsoz, A. Erdem, K. Kerman, D. Ozkan, B. Tugrul, N. Topcuoglu, H. Erken, M. Taylan, *Anal. Chem.* 75 (2003) 2181–2187.
- [44] H. Karadeniz, A. Erdem, A. Caliskan, C.M. Pereira, E.M. Pereira, J.A. Ribeiro, *Electrochem. Commun.* 9 (2007) 2167–2173.
- [45] A. Vaseashta, *J. Mater. Sci.: Mater. Electron.* 14 (10–12) (2003) 653–656.
- [46] L. Authier, C. Grossiord, P. Brossier, B. Limoges, *Anal. Chem.* 73 (2001) 4450–4456.
- [47] E. Palecek, R. Kizek, L. Havran, S. Billova, M. Fojta, *Anal. Chim. Acta* 469 (2002) 73–83.
- [48] J. Wang, G.D. Liu, M.R. Jan, *J. Am. Chem. Soc.* 126 (2004) 3010–3011.
- [49] J. Li, H.T. Ng, A. Cassell, W. Fan, H. Chen, Q. Ye, J. Koehne, J. Han, M. Meyyappan, *Nano Lett.* 3 (2003) 597–602.
- [50] F. Patolsky, C.M. Lieber, *Mater. Today* (2005) 20–28.
- [51] Z. Li, Y. Chen, X. Li, T.I. Kamins, K. Nauka, R.S. Williams, *Nano Lett.* 4 (2) (2004) 245–247.
- [52] M.A. Lapiere-Devlin, C.L. Asher, B.J. Taft, R. Gasparac, M.A. Roberts, S.O. Kelley, *Nano Lett.* 5 (6) (2005) 1051–1055.
- [53] J. Wang, A.-N. Kawde, E. Sahlin, *Analyst* 125 (2000) 219–224.
- [54] E. Palecek, S. Billova, L. Havran, R. Kizek, A. Miculkova, F. Jelen, *Talanta* 56 (2002) 919–930.
- [55] J. Wang, A.-N. Kawde, *Electrochem. Commun.* 4 (2002) 349–352.
- [56] H.X. Ju, Y.K. Ye, J.H. Zhao, Y.L. Zhu, *Anal. Biochem.* 313 (2003) 255–261.
- [57] Y.K. Ye, J.H. Zhao, F. Yan, Y.L. Zhu, X.H. Ju, *Biosens. Bioelectron.* 18 (2003) 1501–1508.
- [58] J. Wang, D. Xu, A.-N. Kawde, R. Polsky, *Anal. Chem.* 73 (2001) 5576–5581.
- [59] M. Dequaire, C. Degrand, B. Limoges, *Anal. Chem.* 72 (2000) 5521–5528.
- [60] H. Cai, Y. Xu, N. Zhu, P. He, Y. Fang, *Analyst* 127 (2002) 803–808.
- [61] N. Zhu, A. Zhang, Q. Wang, P. He, Y. Fang, *Electroanalysis* 16 (2004) 577–582.
- [62] M.T. Castaneda, A. Merkoci, M. Pumera, S. Alegret, *Biosens. Bioelectron.* 22 (2007) 1961–1967.
- [63] M. Pumera, M.T. Castaneda, M.I. Pividori, R. Eritja, A. Merkoci, S. Alegret, *Langmuir* 21 (2005) 9625–9629.
- [64] A. Vaseashta, in: S. Bandopadhyay (Ed.), *Carbon Nanotubes Based Sensor and Devices*, Tata McGrawhill Publishing Co. Ltd., New Delhi, 2004.
- [65] M.T. Castaneda, S. Alegret, A. Merkoci, *Electroanalysis* 19 (7–8) (2007) 743–753.
- [66] D. Hernandez-Santos, M.B. Gonzalez-Garcia, A. Costa-Garcia, *Electroanalysis* 14 (2002) 1225–1235.
- [67] S.G. Penn, L. He, M. Natan, *Curr. Opin. Chem. Biol.* 7 (2003) 609–615.
- [68] S. Schultz, D.R. Smith, J.J. Mock, D.A. Schultz, *Proc. Natl. Acad. Sci.* 97 (2000) 996–1001.
- [69] P. Papakonstantinou, R. Kern, L. Robinson, H. Murphy, J. Irvine, E. McAdams, J. McLaughlin, T. McNally, Fuller. *Nanotube Carbon Nanostruct.* 13 (2004) 91–108.
- [70] M. Yang, M.E. McGovern, M. Thompson, *Anal. Chim. Acta* 346 (1997) 259–275.
- [71] C. Debouck, P.N. Goodfellow, *Nat. Genet. Suppl.* 21 (1999) 48–50.
- [72] J. Hodgson, *Nat. Biotechnol.* 16 (1998) 725–727.

Manufacture and evaluation of carbon nanotube modified screen-printed electrodes as electrochemical tools

Pablo Fanjul-Bolado^a, Paula Queipo^b, Pedro José Lamas-Ardisana^a,
Agustín Costa-García^{a,*}

^a *Departamento de Química Física y Analítica, Universidad de Oviedo, 33006 Oviedo, Asturias, Spain*

^b *Departamento de Física, Universidad de Oviedo, 33007 Oviedo, Asturias, Spain*

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Abstract

Carboxylated multiwalled carbon nanotubes (MWCNT-COOH) dissolved in a mixture of DMF:water were used to modify the surfaces of commercially available screen-printed electrodes (SPEs). The morphology of the MWCNT-COOH and the modified SPEs was characterized by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), respectively. SEM analysis showed a porous structure formed by a film of disordered nanotubes on the surface of the working electrode.

The modification procedure with MWCNT-COOH was optimised and it was applied to unify the electrochemical behaviour of different gold and carbon SPEs by using *p*-aminophenol as the benchmark redox system. The analytical advantages of the MWCNT-COOH-modified SPEs as voltammetric and amperometric detectors as well as their catalytic properties were discussed through the analysis, for instance, of dopamine and hydrogen peroxide. Experimental results show that the electrochemical active area of the nanotube-modified electrode increased around 50%. The repeatability of the modification methodology is around 6% (R.S.D.) and the stability of MWCNT-COOH-modified SPEs is ensured for, at least, 2 months.

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Keywords: Multiwalled carbon nanotubes; Screen-printed electrodes; Cyclic voltammetry; Amperometric detection; Flow injection analysis

1. Introduction

Screen-printing technique is a well-established and simple process for the mass production of single use electrodes and biosensors. These electroanalytical tools combine ease of use, portability and inexpensive manufacture procedure [1–3]. Many research laboratories possess technology for the fabrication of screen-printed electrodes (SPEs) for their own use and some companies are mass producers of SPEs with improved repeatability. In this sense, the manufacture diversity implies the presence on the market of SPEs with different characteristics (i.e., different inks, substrates and heat curing temperatures) that directly influence on their electrochemical behaviour. Some works have shown that the electrochemical behaviour of some

benchmark redox systems (ferricyanide, acetaminophen or hydroquinone) could present great differences depending on these manufacturing components or manufacturing variables [4–6].

The discovery of new nanomaterials opens new paths in the field of sensors. In particular, carbon nanotubes (CNTs) have unique properties that have prompted their use in many different applications [7–9]. The adsorption capacity, the possibility to be functionalized, their ability to promote the electron transfer reactions of a great number of molecules [10,11] and the possibility for miniaturization make CNTs a very attractive material for the further development of electrochemical biosensors. However, one important drawback of CNTs is their difficult solubilization [12]. Currently, organic solvents like DMF or DMSO and aqueous solutions of napon or surfactants are being used to achieve this goal.

Different attempts have been already made to improve the electroanalytical properties of disposable biosensors by combin-

* Corresponding author. Tel.: +34 985103488; fax: +34 985103125.

E-mail address: costa@fq.uniovi.es (A. Costa-García).

ing CNT with SPEs [4,6,13–20]. However, most of them have worked on solving individual analytical troubles. For instance, some glucose biosensors have been developed on CNTs immobilized with glucose oxidase on the surface of homemade SPEs [14] or a catalytic detection of ascorbic acid has been proposed also in a CNT-modified SPE [15]. Similarly, other CNT modifications have been evaluated for the development of enzymatic amperometric thick film biosensors for the determination of nerve agents using homemade SPEs [16–18]. However, to our knowledge only a few useful solutions to develop a production method for CNT-based SPEs has been reported through the fabrication of an ink based on CNTs [4,19,20]. The proposal of Wang et al. [4] is probably based on other thick film CNT devices manufactured for emission display applications [21,22] and implies an unique printing step of the CNT based ink. Other works prepared a CNT/polysulfone composite material that is also printed over a carbon working electrode already manufactured [19,20].

In the present study, an easy and effective CNT modification methodology for SPEs is provided. It is a post-production procedure for improvement the SPE quality that could not increase greatly the price of disposable SPEs. SPEs from diverse manufacturers (thus with different characteristics) were used and electrochemical behaviours were improved getting finally MWCNT-COOH coated SPEs with a similar behaviour. Characterization of the CNT film generated on the surface of the working electrode and stability studies were carried out. This fact is of extremely importance for the future manufacture of biosensors based on the nanostructuration of SPEs with carbon nanotubes.

2. Experimental

2.1. Apparatus

Voltammetric and amperometric measurements were performed with an Autolab PGSTAT 12 (Eco Chemie, The Netherlands) potentiostat/galvanostat interfaced to an AMD K-6 266 MHz computer system and controlled by Autolab GPES 4.8 (software version for Windows 98).

A JEOL JEOL-200 EX-II transmission electron microscope (Japan) was used to characterize MWCNT-COOH. A JEOL JSM-6100 scanning electron microscope (20 kV, Japan) was used to characterize the working electrodes after gold sputtering.

Amperometric flow injection analysis (FIA) was carried out with a simple system that mainly comprised a 12-cylinder Perimax Spetec peristaltic pump (Spetec GmbH, Germany) together with a manual six-port rotary valve Model 1106 (Omnifit Ltd., UK) and a homemade wall-jet flow cell where the SPEs were placed. The flow cell consists on two pieces of methacrylate screwed together; one of those has inlet and outlet flow channels forming an angle of 30°. The SPEs were fixed in between and were easily connected to the potentiostat through an edge connector. A syringe was used to aspirate the samples into the system by filling a loop of 50 µl that was further discharged into the flow system by means of the injection valve.

2.2. Reagents and solutions

Carboxyl-modified multiwalled carbon nanotubes (MWCNT-COOH) were kindly provided by Fundación Inasmet. Samples examined by transmission electron microscopy (TEM) consist of nanotubes with a wide range of diameters, varying from 3 to 15 nm. They are clean, i.e., free of carbon deposits in their walls and no catalyst particles were found. The majority of the nanotubes present open ends as a consequence of the purification and functionalization treatments [23]. A solution was prepared by mixing 1 mg of MWCNT-COOH with 1 ml of a mixture DMF:water (1:1) by sonication for 3 h, enough time to achieve a homogeneous and stable solution. This kind of solution is the main requirement to get a repeatable modifying procedure of SPCEs. The presence of a 50% of water in the solution allows us its use with any kind of SPEs based on any substrate material or ink composition. Pure DMF solutions of CNTs commonly used to modify glassy carbon electrodes are not suitable to be combined with the most of plastic substrates and conductive inks of SPEs.

Hexaammineruthenium(III) chloride, 4-aminophenol, dopamine, hydroquinone, Trizma base and glycine were provided by Sigma–Aldrich (Spain).

Hydrogen peroxide (30%), hydrochloric acid fuming (37%), sulphuric acid (95–97%) and acetic acid glacial (100%) were purchased from Merck (Germany). All other chemicals employed were of analytical reagent grade. Ultrapure water obtained with a Milli-RO 3 plus/Milli-Q plus 185 purification system from Millipore Ibérica S.A was used throughout this work.

2.3. Screen-printed electrodes (SPEs)

Four different types of commercially available SPEs were used. They include a traditional three electrode configuration printed on the same strip and have already been described [24,25]. These screen-printed electrodes present: (1) a carbon disk working electrode with a radius of 2 mm (DropSens ref. 110 and Alderon SPCEs) or with a radius of 1.5 mm (EcoBioServices & Researches); (2) a gold disk working electrode with a radius of 2 mm (DropSens ref. AT 220). The auxiliary electrode was printed on each strip using the same ink of the working electrode and a silver pseudo-reference electrode was always used. They are printed onto plastic (Alderon and EcoBioServices & Researches SPCEs) or alumina (DropSens carbon and gold SPEs) substrate. An insulating layer serves to delimit a working area and electric contacts. The production characteristics of commercial SPEs are regarded by the manufacturers as proprietary information.

Modified SPEs were manufactured by depositing 5 µl of the MWCNT-COOH dispersion on the working electrode surface. The solution was left to dry at room temperature (20 °C) and its absolute evaporation over the working electrode must be assured for the development of the electrochemical assays. Higher evaporation temperature as 37 °C damages totally the SPEs, so this parameter should be controlled.

A specific DropSens connector (ref. DSC) allows the connection of the different SPEs to the potentiostat. Voltammetric measurements on SPEs were performed by placing a 40 μl drop of the corresponding solution to the working area.

3. Results and discussion

3.1. Voltammetric optimization of the SPCEs coating process with MWCNT-COOH

By using a dispersion of MWCNT-COOH in DMF:water (1:1), the carbon working electrode of a commercially available DropSens SPE was modified. An increasing volume of the MWCNT-COOH dispersion was left to dry (at room temperature) on the working electrode surface and then, cyclic voltammograms of dopamine (as model analyte) were recorded. The selection of the redox system and the SPCE was based on the low rate constant of the heterogeneous electron transfer detected. At a scan rate of 50 mV/s, the peak-to-peak potential separation is high ($\Delta E = 450$ mV). Therefore, an irreversible electrochemical process can be assumed for dopamine at the bare electrode. This fact allow us detect easily the improvement of the electronic transfer rate due to the MWCNT-COOH modification.

Fig. 1 displays cyclic voltammograms for 1×10^{-4} M of dopamine for a bare and MWCNT-COOH-modified SPCEs. When the SPCEs were modified with 1 μl of the MWCNT-COOH solution (Fig. 1, curve c), double anodic and cathodic peaks were obtained and the new ones displayed a lower peak-to-peak potential separation. The voltammogram showed two electrochemical processes for dopamine, one on the surface of the original carbon electrode and other on the surface of the MWCNT-COOH. When depositing increasing volumes (2–3 μl) of the MWCNT-COOH solution, the anodic and cathodic currents of the more reversible process increased (Fig. 1, curves d and e) because the surface of the electrode has a large proportion of MWCNT-COOH. Thus, using modification volumes of 4 or 5 μl , cyclic voltammograms of dopamine were characterized by

a unique process due to the absolute modification of the working electrode by the MWCNT-COOH (Fig. 1, curve f).

For the same scan rate (50 mV/s), the peak-to-peak potential separation on a MWCNT-COOH-modified SPCE ($\Delta E = 88$ mV) is lower that that obtained at a bare SPCE ($\Delta E = 450$ mV). These results corroborate an improved electrochemical behaviour for dopamine on the MWCNT-COOH-modified surface in comparison to the bare SPCE, indicating that the MWCNT-COOH had better electron transfer properties. These improved properties were also demonstrated through voltammetric analysis of other important analytes of the catecholamines family (epinephrine and norepinephrine) or hydroquinone as is shown in Fig. 2.

Data obtained from Figs. 1 and 2 demonstrate that 4 or 5 μl of the MWCNT-COOH solution are enough to get a good cover of the original SPCE surface. Such volumes ensure that the original electrochemical surface acts only as an electric contact for the new carbon nanostructured working surface. Increasing the quantity of immobilized nanotubes by drying successive 5 μl drops of MWCNT-COOH solution do not decrease the peak-to-peak potential separation and not increase the faradaic currents. On the other hand, low modification volumes generate working electrode with a mixed surface nature and voltammograms with mixed behaviours.

3.2. SEM and electrochemical characterization of MWCNT-COOH-modified SPCEs

The micro and nanostructure of a MWCNT-COOH-modified SPCE from DropSens was observed by scanning electron microscopy (Fig. 3). The original carbon surface is characterized by a great content of edge graphite particles and a rough surface but in the case of the MWCNT-COOH-modified SPCE a disordered ensemble of carbon nanotubes is visualized. None area of the initial graphite based surface remained uncovered and it looks like a porous carbon electrode. It can be seen that a great number of opened ends of the MWCNT-COOH are exposed to the bulk solution. The ends of these nanotubes are thought to be the major responsible of their catalytic properties due to the greater presence of oxygenated groups like $-\text{COOH}$ groups [26,27]. Also is detected a wide area of the working surface covered by the less reactive sidewalls of the nanotubes. Thus, some works have been developed to get a vertically alignment of nanotubes on the surface of electrodes with the aim of leaving the most of the capped ends oriented to the bulk solution [28,29].

The surface area of the MWCNT-COOH-modified SPCE should be greater than the area of the unmodified SPCE due to its roughly nanostructure. To assure this statement the electrochemical surface area of these electrodes was calculated using the Randle-Sevcik equation for a reversible electrochemical process under diffusive control ($T^a = 25^\circ\text{C}$):

$$i_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$$

where n is the number of electrons involved in the redox reaction, A the electroactive area of the electrode (cm^2), D the diffusion coefficient of the molecule in solution ($8.06 \times 10^{-6} \text{ cm}^2/\text{s}$ for

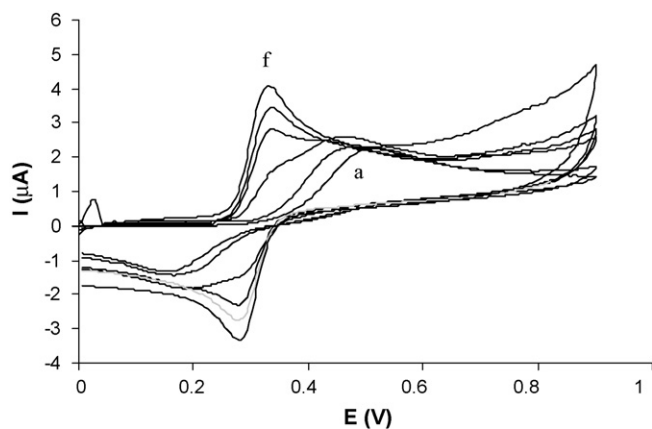


Fig. 1. Cyclic voltammograms of dopamine 10^{-4} M in HCl 0.01 M at a DropSens SPCE: (a) naked, (b) modified with 2 μl of DMF:water (1:1), and modified with (c) 1 μl , (d) 2 μl , (e) 3 μl and (f) 4 μl of MWCNT-COOH solution in DMF:water.

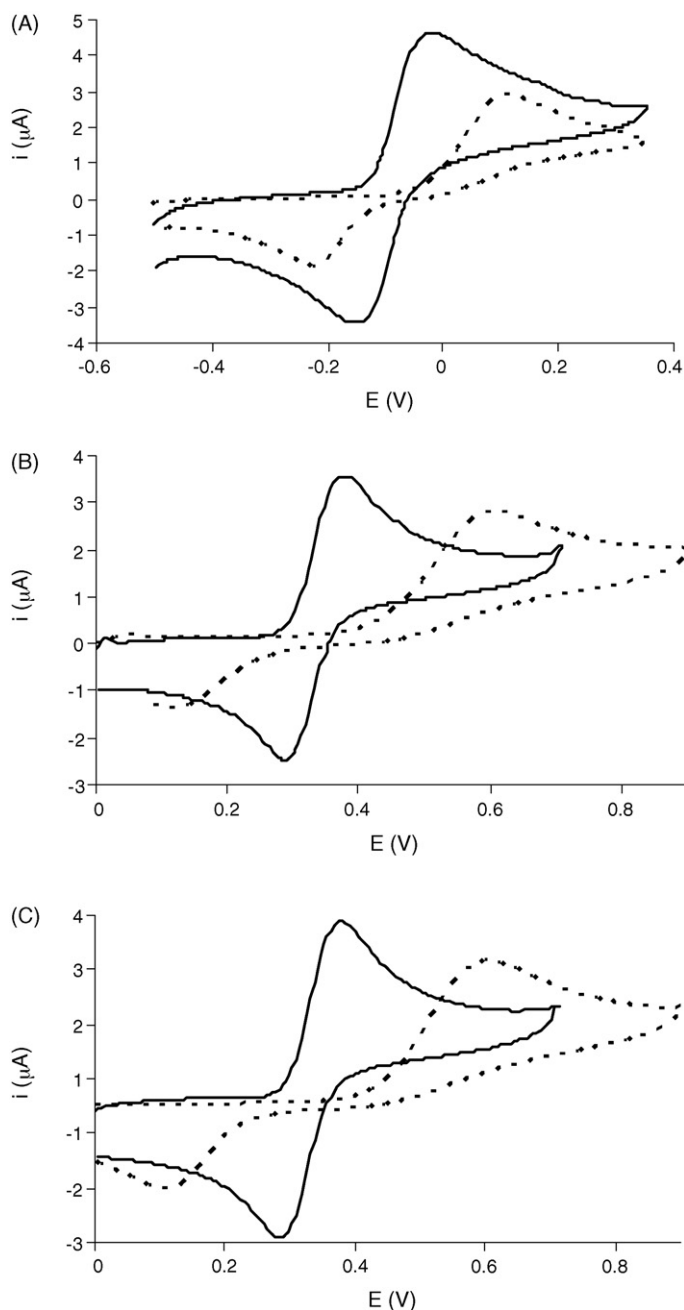


Fig. 2. Cyclic voltammograms of 10^{-4} M (A) hydroquinone in acetate buffer 0.1 M pH 5.0, (B) epinephrine in HCl 0.01 M and (C) norepinephrine in HCl 0.01 M at a DropSens bare SPCE (dashed line) and at a DropSens SPCE-modified with 4 μ l of MWCNT-COOH solution (solid line).

hexaammineruthenium(III) in 0.1 M KCl [30]), C the concentration of the analyte molecule in the solution (mol/cm^3) and v is the scan rate (V/s).

Cyclic voltammograms for 1×10^{-3} M hexaammineruthenium(III) in 0.1 M KCl were registered at different v (from 50 to 1000 mV/s) with the two kinds of electrodes. The peak-to-peak potential separation was constant and linear relationships between the cathodic peak current and the square root of the scan rate were achieved. From the slope of these equations the electrochemical surface area was calculated. The electrochemical area of the bare SPCE from DropSens was $1.09 \times 10^{-5} \text{ m}^2$ and the equivalent radius was 1.86 mm. Taking into account that the geometric radius of the working electrode is 2 mm, this result shows that the rough carbon is partially passivated due to surface contaminants or due to the organic binder of the carbon ink. The SPCE coated with MWCNT-COOH have an active surface area of $1.59 \times 10^{-5} \text{ m}^2$ and an equivalent radius of 2.2 mm. Therefore, experimental results reveal that the modification with nanotubes increases the electrochemical area of the SPCE in around a 50%.

The double-layer capacitance (C_{dl}) of the two electrodes was also estimated due to the importance of the capacitive currents to the electroanalytical applications of the MWCNT-COOH-modified electrodes. Cyclic voltammograms were obtained for solutions containing only the supporting electrolyte (0.1 M KCl). At a fixed potential far removed from the switching potential (in this case -0.3 V), the capacitive current $|i_c|$ is given by the equation:

$$|i_c| = AC_{\text{dl}}v$$

where v is the scan rate (V/s), A the effective electrochemical area (cm^2) previously calculated and C_{dl} is the double-layer capacitance (F/cm^2). According to the equation, i_c was plotted versus v (varied between 50 and 1000 mV/s) and a linear relation was attained for the two electrodes investigated here (correlation coefficients greater than 0.998 were obtained). From the slope of the linear plot a $C_{\text{dl}} = 2.21 \text{ } \mu\text{F}/\text{cm}^2$ was obtained for the bare SPCE and a $C_{\text{dl}} = 35.2 \text{ } \mu\text{F}/\text{cm}^2$ was found for the MWCNT-COOH-modified SPCE. For analytical purposes, such an increase of the C_{dl} for the modified SPCE showed us that will be very difficult to achieve lower detection limits at this electrode by cyclic voltammetry.

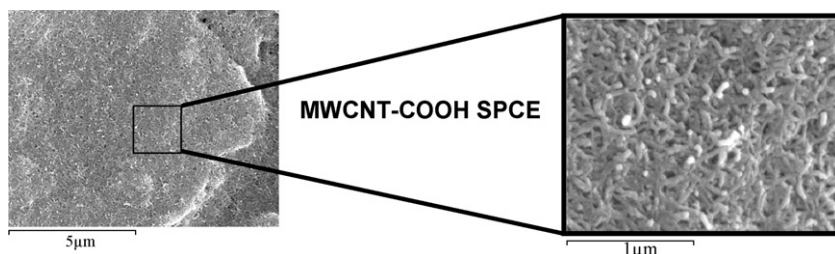


Fig. 3. SEM image of a MWCNT-COOH-modified SPCE.

3.3. Electroanalytical comparison of MWCNT-COOH-modified SPCEs and bare SPCEs

3.3.1. Dopamine detection

The determination of dopamine was carried out by cyclic voltammetry at a scan rate of 50 mV/s. With a bare DropSens SPCE a calibration curve was obtained with a linear dynamic range from 10^{-6} to 5×10^{-4} M, however, using the MWCNT-COOH-modified electrode the linear dynamic range comprises concentrations between 5×10^{-6} and 5×10^{-3} M (data not shown). Therefore, the previously mentioned increase of the capacitive currents in the modified electrode is the main cause of the loss of analytical signal for detectable concentrations at the bare electrode. Also a decrease of the interelectrode repeatability (for 10^{-4} M of dopamine) from 4 to 6% was detected by cyclic voltammetry due to the MWCNT-COOH modification procedure of the SPEs.

A linear relationship between the anodic peak current and the square root of the scan rate (between 20 and 250 mV/s) demonstrated that the dopamine process is diffusion controlled. Therefore, the analytical performance comparison of both kinds of SPEs was developed as amperometric detectors (for dopamine) in a FIA system. In this case a potential is fixed during the electrochemical measurements and the contribution of the capacitive current is minimised. The slope, intercept, regression coefficient and linear dynamic range (LDR) of the calibration plots carried out are reported in Table 1. In this case, the LDR are quite similar although a greater slope appears as an advantage of the modified SPEs.

By using this amperometric detection methodology the MWCNT-COOH-modified SPCEs enhanced the noise of the base line at the diagram, probably due to its porous structure and to the increased roughness of the working surface electrode. This fact does not allow us to quantify lower concentrations of dopamine than 2.5×10^{-7} M (flow rate 1.8 ml/min). At this point the best advantage of the MWCNT-COOH-modified SPCEs for their use as electrochemical detectors of dopamine in a FIA system is the decrease of the potential detection. This potential was optimised through the corresponding hydrodynamic curves. While in the case of the bare SPE a +0.8 V potential was needed, when a modified SPCE was used a +0.6 V detection potential was enough to obtain the highest analytical signal. Employing optimized conditions in both cases a R.S.D. below 5% was achieved for 10 measurements of 1×10^{-6} M of dopamine, thus a good intraelectrode repeatability of the MWCNT-COOH-modified SPCEs is assured for their use in a flow injection analysis system.

3.3.2. H_2O_2 and NADH detection

These two compounds were taken also as model analytes for the evaluation of the electroanalytical properties of MWCNT-COOH-modified SPCEs because its measurement is of considerable interest to the operation of oxidase- and dehydrogenase-based amperometric biosensors.

Fig. 4 displays amperometric responses for hydrogen peroxide in a FIA system as recorded at a DropSens MWCNT-COOH-modified electrode at +0.6 V. Although ox-

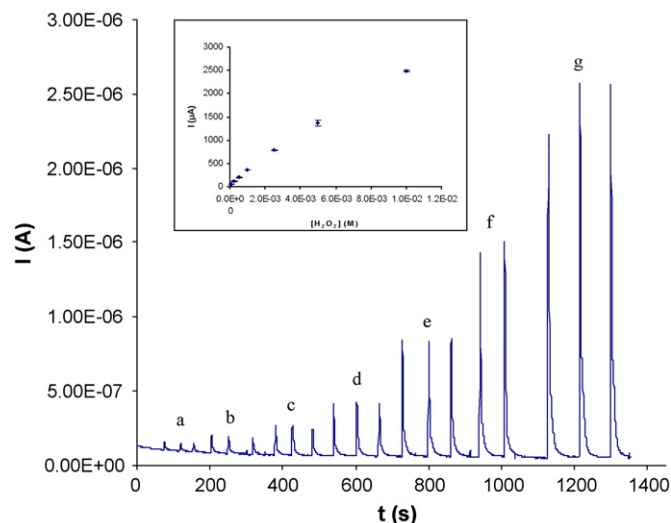


Fig. 4. Flow injection amperometric signals for hydrogen peroxide (a) 10^{-4} M, (b) 2.5×10^{-4} M, (c) 5×10^{-4} M, (d) 10^{-3} M, (e) 2.5×10^{-3} M, (f) 5×10^{-3} M and (g) 10^{-2} M, at the MWCNT-COOH-modified DropSens SPCE. E_{det} , +0.6 V; flow rate, 2.2 ml/min; electrolyte, phosphate buffer 0.05 M pH 7.4. Inset shows the resulting calibration plot for hydrogen peroxide.

idation currents could be detected at low detection potentials for 5×10^{-3} M of hydrogen peroxide, +0.6 V was selected to calibrate from 10^{-4} to 10^{-2} M of hydrogen peroxide. In contrast, amperometric response was not observed for the bare analogous DropSens SPCE at this detection potential, what demonstrates the electrocatalytic properties of MWCNT-COOH-modified SPCEs.

For the analytical determination of NADH, Alderon Biosciences SPCEs were used. With this different kind of SPCEs based on a plastic substrate the electrocatalytic properties of MWCNT-COOH are also clearly detected. Fig. 5 (inset) compares cyclic voltammograms for 10^{-3} M of NADH at a bare (dashed line) and MWCNT-COOH-modified SPCE (solid line). The oxidation current for NADH starts at potential higher than +0.65 V at the bare electrode while at the modified electrode it

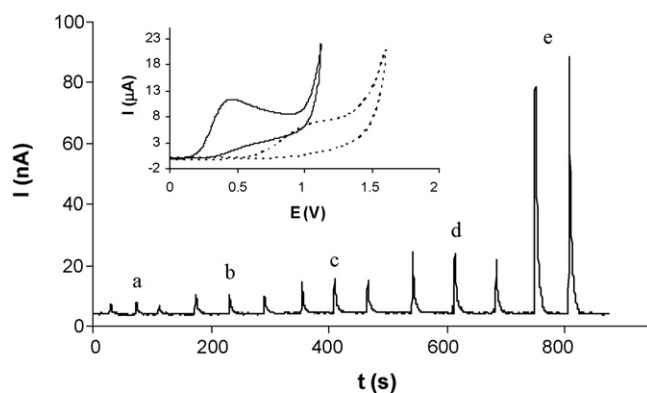


Fig. 5. Flow injection amperometric signals for NADH (a) 10^{-6} M, (b) 2.5×10^{-6} M, (c) 5×10^{-6} M, (d) 10^{-5} M and (e) 5×10^{-5} M, at the MWCNT-COOH-modified Alderon SPCE. E_{det} , +0.45 V; flow rate, 2.2 ml/min; electrolyte, phosphate buffer 0.05 M pH 7.4. Inset shows cyclic voltammograms for 10^{-3} M NADH at unmodified (dashed line) and MWCNT-COOH-modified (solid line) Alderon SPCE electrodes.

Table 1
Characteristics of the calibration plots obtained for the amperometric detection of dopamine in a FIA system at a bare DropSens SPCE ($E_{\text{det}} = +0.8$ V) and at a MWCNT-COOH-modified DropSens SPCE ($E_{\text{det}} = +0.6$ V)

	Slope (nA/M) ($\times 10^7$)	Intercept (nA)	r^2	LDR (M)
SPCE	2	−6.09	0.999	2.5×10^{-7} – 10^{-4}
MWCNT-COOH SPCE	4	−5.48	0.999	2.5×10^{-7} – 10^{-4}

Flow rate 1.8 ml/min; carrier stream HCl 0.01 M.

starts at +0.2 V. This result show that immobilized MWCNT-COOH promote in a very efficient way the electron transfer between the NADH and this kind of material electrode. This electrocatalytic activity allows low potential (+0.45 V) amperometric determination of NADH in a flow injection analysis system as it is also showed in Fig. 5. The corresponding calibration plot was linear between 10^{-6} and 5×10^{-5} M of NADH and followed the equation:

$$i \text{ (nA)} = 10^6 [\text{NADH}](\text{M}) + 3.3, \quad r^2 = 0.998$$

The limit of detection, calculated as the concentration corresponding to three times the standard deviation of the intercept, was 7×10^{-7} M.

Fouling of the electrode by the oxidation product of NADH was not detected using NADH concentrations corresponding to the linear dynamic range, higher concentrations than 10^{-4} M for NADH already produced a signal decrease after successive injections.

3.4. MWCNT-COOH modification of SPEs from different manufacturers

Four different SPEs were used to be modified with the MWCNT-COOH solution: (1) a SPCE from DropSens, (2) a SPCE from Alderon Biosciences, (3) a SPCE from EcoBioServices&Researches and (4) a gold SPE from DropSens. These four electrodes comprised a heterogeneous group with alumina and plastic substrates and different carbon or gold inks. As redox system, a compound that showed quite different redox behaviour depending on the electrodic surface, like *p*-aminophenol was selected. The detection of *p*-aminophenol is interesting for future biosensors based on the detection system alkaline phosphatase/*p*-aminophenyl phosphate. As is indicated in Fig. 6A, the response in the *p*-aminophenol solution goes from a reversible system on DropSens SPCE (dashed black line) to an irreversible process on the Alderon SPCE (dashed grey line). In order to facilitate the comparison, each voltammogram was normalized versus its maximum anodic current.

After modification with 5 μ l of the MWCNT-COOH dispersion all four electrodes gave quite similar good results (Fig. 6B). Reversible waves were observed for all four MWCNT-COOH-modified electrodes examined for *p*-aminophenol, indicating that the nanotube material remain its good electron transfer properties on all these four supporting surfaces. These results also corroborate that the modification solution and methodology could be used with gold or carbon screen-printed electrodes and with SPEs based on plastic or ceramic substrates. Therefore, a useful universal modification of any kind of SPEs with

MWCNT-COOH is proposed to improve their electrochemical properties.

3.5. Stability studies of the MWCNT-COOH-modified SPEs

The stability of the MWCNT-COOH-modified SPEs stored at room temperature and in a dry place was studied by carrying out cyclic voltammograms of dopamine (10^{-4} M) and hydroquinone (10^{-4} M). The measured anodic peak current remains stable during almost 2 months without any loss on intensity. This fact mainly demonstrates the stability of nanotubes and the good storage conditions avoiding any surface contamination that could decrease the electrochemical active area of the working electrode. The peak-to-peak potential separation

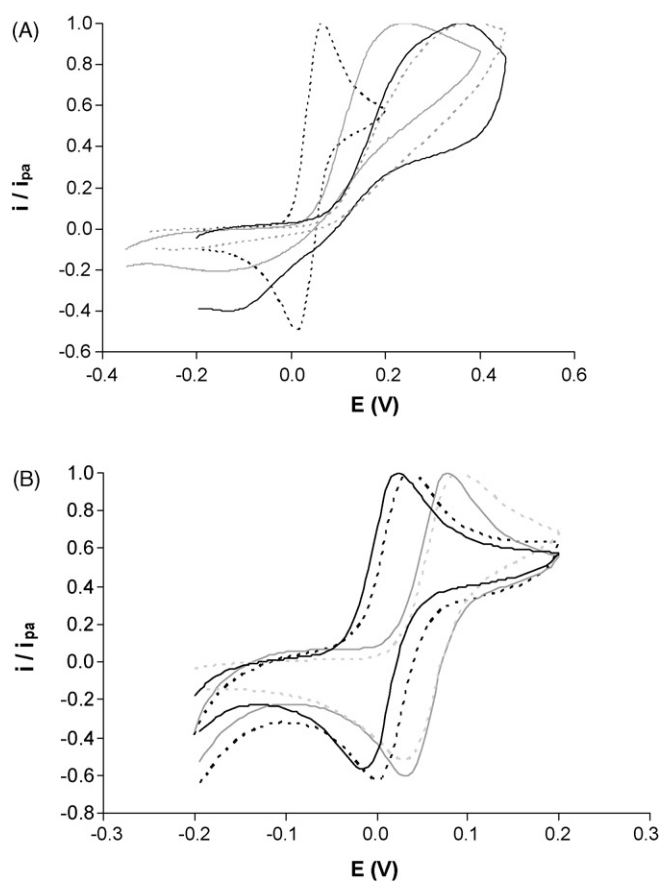


Fig. 6. Normalized cyclic voltammograms of 10^{-4} M *p*-aminophenol in a Tris-glycine 0.5 M pH 9.4 solution at four different (A) naked and (B) MWCNT-COOH-modified SPEs. Scan rate 50 mV/s. (1, solid black line) DropSens Gold SPE (ref. AT220); (2, dashed black line) DropSens carbon SPE (ref. 110); (3, dashed grey line) Alderon carbon SPE; (4, solid grey line) EcoBioServices & Researches carbon SPE.

increases during the first 2 weeks from 54 to 79 mV, and then remains stable in this value during almost 2 months. This assay informed us on an initial little loss of catalytic and electronic transfer properties during the first storage days that are then stabilized.

Also the stability of the MWCNT-COOH dispersion on DMF:water (1:1) was probed by modifying SPEs during a period of 3 months. On this time, the dispersion of nanotubes remains as a homogeneous solution without the precipitation of any solid in the mixture and the modified SPEs manufactured with this solution presented similar analytical and electronic transfer properties. To our knowledge these stability studies that are essential for future development of biosensors on these devices are reported for first time at this work. Besides, manufacture repeatability could be improved by using automatic microvolume dispensers mainly used in the mass production of glucose biosensors.

4. Conclusions

A simple MWCNT-COOH modification procedure for gold or carbon based SPEs printed on plastic or alumina substrate has been developed. The post-production procedure that uses 1 mg of MWCNT-COOH for the modification of 200 SPEs is a cheap way to improve the quality of these disposable devices. The potentiality of the new platform (MWCNT-COOH-modified SPE) was clearly demonstrated by using different analytes and techniques. The nanotube-modified electrodes presented better electron transfer and catalytic properties and enhanced electrochemical active area than conventional SPEs. However, the analytical performance of these electrodes as voltammetric or amperometric detectors is limited in order to obtain lower detection limits.

The stability of modified SPEs is assured at least for 2 months stored in a dry place at room temperature. Furthermore, their mechanical strength allows its use as detectors in flow systems. Once the electrochemical behaviour of these MWCNT-COOH-modified SPEs has been evaluated, and mainly once a good stability and manufacture repeatability has been achieved, future efforts will be focused on the development of enzyme, immuno and DNA based biosensors using these MWCNT-COOH-modified SPEs as transducers.

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Dear Joe,

From these lines I would like to congratulate you for your excellent work. I want to thank you for your example, your efforts

and your generosity, so not very often recognized, and I wish you a future full of happiness.



References

- [1] J.P. Hart, S.A. Wring, *Electroanalysis* 6 (1994) 617.
- [2] J.P. Hart, S.A. Wring, *TRAC* 16 (2) (1997) 89.
- [3] K.C. Honeychurch, J.P. Hart, *TRAC* 22 (7/8) (2003) 456.
- [4] J. Wang, M. Musameh, *Analyst* 129 (2004) 1.
- [5] Y. Yao, K.-K. Shiu, *Anal. Bioanal. Chem.* 387 (2007) 303.
- [6] M. Trojanowicz, A. Mulchandani, M. Mascini, *Anal. Lett.* 37 (15) (2004) 3185.
- [7] A. Merkoçi, M. Pumera, X. Llopis, B. Pérez, M. del Valle, S. Alegret, *TRAC* 24 (9) (2005) 826.
- [8] J. Wang, G. Liu, M.R. Jan, *JACS* 126 (2004) 3010.
- [9] J. Robertson, *Mater. Today* October (2004) 46.
- [10] H. Luo, Z. Shi, N. Li, Z. Gu, Q. Zhuang, *Anal. Chem.* 73 (2001) 915.
- [11] M. Musameh, J. Wang, A. Merkoçi, Y. Lin, *Electrochem. Commun.* 4 (2002) 743.
- [12] J. Wang, M. Musameh, Y. Lin, *JACS* 125 (2003) 2408.
- [13] C.-H. Lee, S.-C. Wang, C.-J. Yuan, M.-F. Wen, K.-S. Chang, *Biosens. Bioelectron.* 22 (6) (2007) 877.
- [14] W.-J. Guan, Y. Li, Y.-Q. Chen, X.-B. Zhang, G.-Q. Hu, *Biosens. Bioelectron.* 21 (2005) 508.
- [15] Y. Sha, L. Qian, Y. Ma, H. Bai, X. Yang, *Talanta* 70 (2006) 556.
- [16] Y. Lin, F. Lu, J. Wang, *Electroanalysis* 16 (1/2) (2004) 145.
- [17] K.A. Joshi, J. Tang, R. Haddon, J. Wang, W. Chen, A. Mulchandani, *Electroanalysis* 17 (1) (2005) 54.
- [18] K.A. Joshi, M. Prouza, M. Kum, J. Wang, J. Tang, R. Haddon, W. Chen, A. Mulchandani, *Anal. Chem.* 78 (1) (2006) 331.
- [19] S. Sánchez, M. Pumera, E. Cabruja, E. Fábregas, *Analyst* 132 (2007) 142.
- [20] S. Sánchez, M. Pumera, E. Fábregas, *Biosens. Bioelectron.* 31 (2007) 332.
- [21] D. Lee, M. Yi, H. Jung, W. Seo, J. Park, H. Chun, N. Koh, *Thermec'2003*, 426–4, 2003, p. 2297.
- [22] Y. Li, C. Zhu, X. Liu, *Diamond Relat. Mater.* 11 (2002) 1845.
- [23] S. Goyanes, G.R. Rubiolo, A. Salazar, A. Jimeno, M.A. Corchera, I. Mondragón, *Diamond Relat. Mater.* 16 (2007) 412.
- [24] P. Fanjul-Bolado, M.B. González-García, A. Costa-García, *Talanta* 64 (2004) 452.
- [25] R. García-González, M.T. Fernández-Abedul, A. Costa-García, *Elect. Acta*, in press.
- [26] A. Chou, T. Böcking, N.K. Singh, J.J. Gooding, *Chem. Commun.* (2005) 829.
- [27] C.E. Banks, T.J. Davies, G.G. Wildgoose, R.G. Compton, *Chem. Commun.* (2005) 842.
- [28] D. Chattopadhyay, I. Galeska, F. Papadimitrakopoulos, *J. Am. Chem. Soc.* 123 (2001) 9451.
- [29] X.-J. Huang, H.-S. Im, O. Yarinaga, J.-H. Kim, D.-Y. Jang, D.-H. Lee, H.-S. Kim, Y.-K. Choi, *J. Electroanal. Chem.* 594 (2006) 27.
- [30] C.G. Zoski, *Electroanalysis* 14 (2002) 1041.

Preparation and characterization of carbon paste micro-electrode based on carbon nano-particles

Samo B. Hočevár*, Božidar Ogorevc

Analytical Chemistry Laboratory, National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia

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Abstract

The present paper demonstrates the preparation and characterization of micro-electrodes based on carbon paste which is composed of carbon nano-particles with an average diameter of 30 nm and binding oil. The carbon paste electrode material is encased in pulled glass capillaries ranging in diameter from several tens down to less than ten micro-meters ($r = 4.5 \mu\text{m}$). Manipulation of the carbon paste micro-electrode (CPME) was accomplished via newly developed piston-driven system which construction and related problems are presented. Several parameters influencing the CPME performance including carbon paste composition and its electrochemical activation/preconditioning were investigated. Basic electrochemical behavior and properties were examined using typical redox system, i.e. potassium hexacyanoferrate. Applicability of the proposed carbon paste micro-electrode is illustrated by measuring some potentially interesting organic and inorganic analytes such as dopamine, ascorbic acid and selected heavy metals.

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Keywords: Carbon paste micro-electrode; Nano-particles; Voltammetry; Stripping chronopotentiometry; Dopamine; Ascorbate; Lead; Cadmium

1. Introduction

Since their first introduction in 1958 by Adams [1], carbon paste electrodes have been extensively employed in various electrochemical detection schemes due to their simple and fast preparation, facile surface renewing, biocompatibility, non-toxic character, and relatively low-background characteristics [2]. Furthermore, carbon paste-based electrode material is extremely attractive for its subsequent bulk modification with different modifiers, e.g. electrocatalysts and/or enzymes by simply mixing them into the carbon paste matrix [3–6]. In addition, surface modification of carbon paste electrodes with thin metal films [7], (bio)recognition elements [8,9] and/or protective membranes [6,10,11] is also widely applicable, although their bulk modification is more convenient and common. Mostly, carbon paste electrodes are used as conventional size electrodes in combination with different holders which facilitate their manipulation and renewing of the electrode surface. There are several reports about their miniaturization, where the carbon paste was intro-

duced into small glass or Teflon capillaries with diameters from 75 to 100 μm [6,12–14]. To the best of our knowledge, there is only one work reporting the preparation of carbon paste micro-electrode with its radius of 10 μm [15]. One of the emerging problems accompanying miniaturization procedures is the size of carbon particles employed in the carbon paste and manipulation of the carbon paste micro-electrode. Conductive nano-materials and in particular carbon nano-tubes along with their attractive mechanical, electronic and electrocatalytic properties offer numerous possibilities for introducing new schemes in electrochemical sensor (micro)design, among which, carbon paste electrodes and micro-electrodes certainly represent a significant part [16–19].

Micro- and ultra-micro electrodes have become of increasing importance in electroanalysis, particularly in biomedical and environmental analysis, not only because they offer convenient measurements in extremely small sample volumes and at micro-locations, but also due to their inherently advantageous characteristics [20,21], such as prevailing radial diffusion, thin double layer, low capacitive currents and low Ohmic drop, fast mass flux and high current densities, significantly enhanced signal to noise ratio, high temporal and spatial resolution, immunity to convection, ready measurements in highly resistive

* Corresponding author. Tel.: +386 1 4760 214; fax: +386 1 4760 300.
E-mail address: samo.hocevar@ki.si (S.B. Hočevár).

media, enabled two electrode configuration, and nevertheless relatively low-cost [22,23]. Thus, combining miniaturization strategies with such a flexible electrode material as carbon paste [2] together with advantageous electronic, physical and electrochemical features of carbon nano-particles [24] is therefore very promising, in particular considering development of sensitive electrochemical micro-sensors and biosensors for their potential application in more challenging environments, e.g. in vivo and in vitro molecular physiological studies, single cell secretion monitoring, measurements in the absence of a supporting electrolyte, etc. However, progress in this direction has been mainly restrained by the availability of low-size carbon particles and/or the degree of micro-fabrication technology.

In this work we report on the usage of carbon nano-particles (Carbon Black) as an attractive electrode nano-material which also enables the circumvention of size-related problems during tailoring carbon paste micro-electrodes. The construction of a simple piston-driven micro-manipulator is also presented which serves as a holder for the carbon paste micro-electrode and, at the same time, for simple manipulation/renewal of the electrode surface. The electrochemical characterization and performance of the above-mentioned micro-electrode is presented in the model solution containing dopamine, ascorbate and some selected heavy metals.

2. Experimental

2.1. Apparatus

Cyclic voltammetric, square-wave voltammetric and stripping chronopotentiometric measurements were carried out using a modular electrochemical system Autolab (Eco Chemie, Utrecht, The Netherlands) equipped with PGSTAT12 and ECD modules driven by GPES software (Eco Chemie). A three-electrode configuration consisted of a carbon paste micro-electrode (CPME), Ag/AgCl/KCl(satd.), and a platinum wire as the working, reference and counter electrode, respectively. Electrochemical experiments were carried out in a Metrohm-type voltammetric cell (10–20 ml), placed in a laboratory made Faraday cage, at conditioned room temperature ($23 \pm 1^\circ\text{C}$).

2.2. Reagents

Potassium hexacyanoferrate (HCF) was provided by Fluka, dopamine by Sigma and ascorbic acid by Kemika. Bismuth(III) stock solution (1000 mg L^{-1} in 5 wt.% HNO_3) was supplied by Aldrich, while both lead(II) and cadmium(II) stock solutions (1000 mg L^{-1} in 5 wt.% HNO_3) were purchased from Merck. All other chemicals employed in this work were of analytical grade purity and used as received. Water used to prepare all solutions throughout the work was first deionized and then further purified via an Elix 10/Milli-Q Gradient unit (Millipore, Bedford, USA). Acetate buffer solution (0.05 M, pH 4.5) was prepared by mixing appropriate amounts of acetic acid (Merck) and sodium acetate (Merck). A 0.1 M phosphate buffer solu-

tion was prepared by mixing corresponding amounts of 0.1 M aqueous solutions of disodium hydrogen phosphate (Merck) and sodium dihydrogen phosphate (Kemika) to obtain the pH of 7.4.

2.3. Preparation of carbon paste electrode and micro-electrode

If not otherwise specified, the carbon paste was prepared by thoroughly homogenizing an adequate amount of usually 20% of extremely conductive carbon nano-particles (Carbon Black, Printex XE 2, Degussa, Germany) and paraffin liquid (Uvasol, Merck) using a pestle and mortar. Finally, the paste mixture was packed into the pulled glass capillary with radius down to $4.5\text{ }\mu\text{m}$ of piston-driven micro-electrode holder (Fig. 1). After mechanically removing the redundant paste with a filter paper or a jet of water followed by the electrochemical preconditioning, the electrode was ready to use. For optimization experiments, the carbon paste material was packed into conventional size carbon paste electrode holder with inner diameter of 1 mm.

2.4. Measurement procedures

Cyclic voltammetric measurements for the characterization of CPMEs were performed in the solution containing 0.1 M potassium chloride together with 1 mM potassium hexacyano-

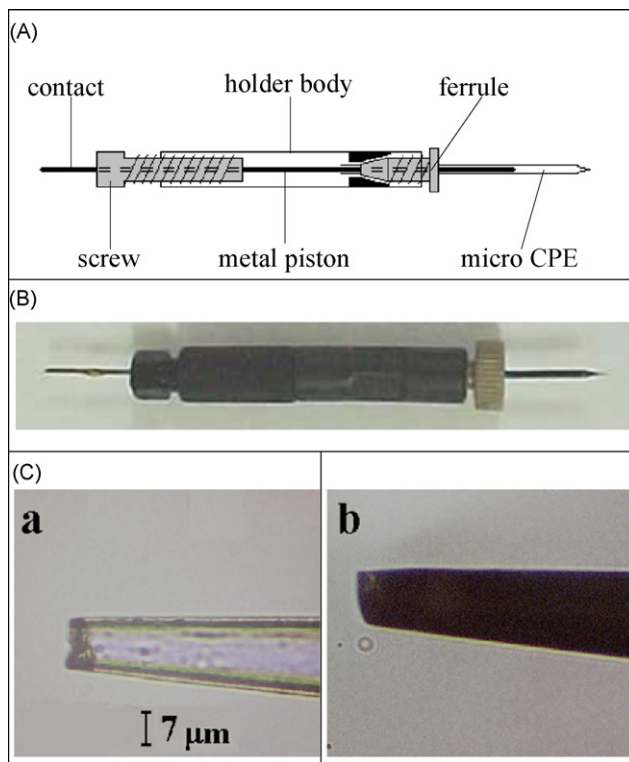


Fig. 1. Schematic picture (A) and photography (B) of newly developed piston-driven micro-manipulator used as a carbon paste micro-electrode holder, and microscopic photography (C) of empty (a) and carbon paste filled (b) pulled end of glass capillary/micro-electrode.

ferrate as a typical redox system by scanning potential in the range from +0.5 to 0.0 V. Other cyclic voltammetric and square-wave voltammetric measurements of organic analytes were carried out in a 0.1 M phosphate buffer solution with pH 7.4.

Stripping chronopotentiometric measurements were conducted in a 0.05 M acetate buffer solution with pH of 4.5 in the presence of dissolved oxygen. The deposition potential of usually -1.2 V was applied to the working electrode for 120 s. During the deposition period the solution was stirred, and after 15 s of equilibration, a chronopotentiogram was recorded by applying the oxidative current of $0.05 \mu\text{A}$ between working and counter electrode. The scan was terminated at $+0.15$ V. Prior to next measurement, the working electrode was held for 30 s at the potential of $+0.3$ V in a stirred solution.

3. Results and discussion

Preliminary experiments have proven that handling a carbon paste micro-electrode (CPME) can be extremely tedious and troublesome without an appropriate holder, which would offer convenient manipulation and renewal of the electrode surface and would at the same time serve as electrode housing. Having this in mind, we developed a new electrode holder, which is based on a piston-driven micro-manipulator system presented in schematic Fig. 1A and as photography in Fig. 1B. The whole arrangement is constructed from parts which are usually used as accessories in flow-system techniques, such as ferrules, unions and end plugs. The holder body (Fig. 1A) consists of two fittings (unions) with a ferrule on one side and a plastic screw (end plug) with embedded copper wire on the other side. The ferrule serves as a holder for glass capillary which is filled with carbon paste, while the screw on the other side of the body serves as a manipulator for copper wire. The copper wire, with the same diameter as the inner diameter of the non-pulled part of glass capillary, provides renewal of the electrode surface by pushing a carbon paste toward capillary's pulled end and at the same time assures an electric contact with the instrument. The pulled end of glass capillary (Fig. 1C), which is prepared by fine pulling technique using a capillary puller followed by precise polishing using micro-electrode beveller, also defines the size of the CPME. When a portion of carbon paste is pushed through the capillary with aim at renewing the electrode surface, the pulled end of glass capillary is gently brushed with a filter paper or flushed with a jet of water from plastic squeeze bottle. The glass capillary is filled with a carbon paste material through its wider (non-pulled) end.

Another problem emerged during preparation of the carbon paste micro-electrode is associated with the size of electrode material, i.e. carbon particles, which are mixed together with selected binding oil forming an appropriate carbon paste electrode material. To circumvent this size-limiting issue, we employed carbon nano-particles (Carbon Black, Printex XE 2, Degussa, Germany) with average diameter of 30 nm which have been proven as a very suitable, extremely conductive and relatively inexpensive electrode material. To provide the best possible operation of the CPME, we optimized several

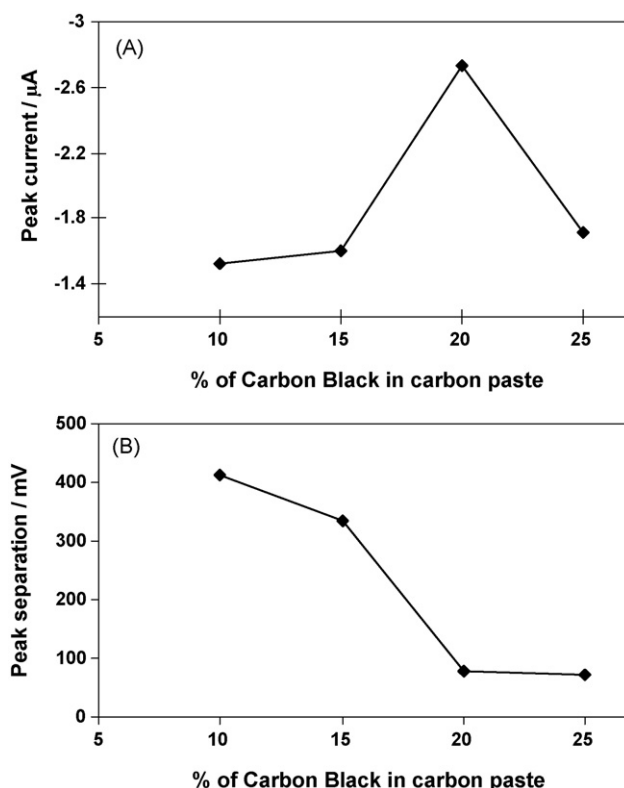


Fig. 2. Effect of Carbon Black loading (%) in carbon paste upon the peak current (A) and peak separation (B) at carbon paste electrode with radius of 0.5 mm in the solution of 1 mM HCF containing 0.1 M KCl. Cyclic voltammetry with a scan rate of 100 mV s^{-1} , initial potential, 0.5 V; final potential, 0.0 V. Preconditioning for 1 s at $+1.4$ V.

parameters, such as composition of carbon paste material, electrochemical preconditioning of electrode surface, etc. In this preliminary part, aimed at shortening and simplifying the optimization step, we carried out the experiments employing a conventional size carbon paste electrode with a diameter of 1 mm. Fig. 2 demonstrates the effect of carbon particles-to-binding oil ratio upon the cyclic voltammetric signal for 1 mM HCF as a typical redox system for common testing of new electrode materials. Before testing the electrochemical performance of carbon paste material, the electrode was preconditioned in the solution of 0.1 M KCl using a constant potential of $+1.4$ V for 1 s. Experiments which are related to this electrochemical preconditioning step are discussed below. As can be seen from Fig. 2A, the highest cathodic current signal/peak is obtained at the electrode surface containing 20% of carbon nano-particles and 80% of binding oil. Lower current in the case of even higher amount of carbon nano-particles, i.e. 25%, can be ascribed to lower consistency of the carbon paste at this ratio also observed as crushing during its preparation. We examined the effect of carbon paste composition upon the peak separation between anodic and cathodic current peaks corresponding to HCF redox system shown in Fig. 2B. The peak separation characteristics indicating the electron transfer kinetics (i.e. the degree of reversibility) are of great significance in determining the electrochemical detection capabilities. We observed that, also in accordance with experiments described

in Fig. 2A, the highest electrochemical performance, i.e. the lowest peak separation, was achieved when the ratios of carbon particles-to-binding oil were 25:75 and 20:80. On the basis of this study and results from Fig. 2A, the corresponding ratio of 20:80 was selected and used in the following experiments.

As already mentioned above, we noticed considerable improvement in electrochemical signal, if the surface of carbon paste was subjected to electrochemical preconditioning prior to its use. Hence, we examined the parameters, which might exhibit the pronounced effect upon the electroanalytical performance of carbon paste electrode based on Carbon Black nano-particles, i.e. preconditioning potential, preconditioning time and the composition of preconditioning solution. The effect of preconditioning potential upon the signal height for 1 mM HCF is demonstrated in Fig. 3A. A stepwise increase of preconditioning potential in the range from 0.0 to +0.8 V did not elicit any significant improvement with respect to the electrochemical behavior of CPME. However, after sequential increasing the potential from +0.8 to +1.6 V, we observed a substantial improvement of the signal corresponding to the reduction and oxidation of HCF. Since the preconditioning at +1.6 V yielded distinctly distorted signals, we corroborated the potential of +1.4 V as an optimum. Furthermore, we studied the influence of preconditioning time upon the signal height and we concluded that the most adequate preconditioning time is in the range from

1 to 3 s as depicted in Fig. 3B. With longer preconditioning times, the electrode response commenced to decrease and after prolonged preconditioning the distorted voltammograms were observed. Since the preconditioning is carried out at relatively high potential of +1.4 V, we can assume that prolonged exposure can induce additional over-oxidation of the binding oil and/or electrode surface reflecting attenuated conductivity of the electrode and in addition, longer exposure to the aforesaid potential might induce oxygen evolution which partially covers and thus decreases the active electrode surface. However, the application of higher potential (+1.4 V) is necessary (Fig. 3A) as the carbon paste material includes relatively high amount of binding oil and due to the inherent surface characteristics of Carbon Black material. Another parameter with a pronounced effect upon the electrochemical performance is the medium in which the preconditioning takes place. To further improve the performance of newly developed carbon paste electrode, we checked the impact of different electrolytes added to the preconditioning solution, i.e. NaNO_3 , LiClO_4 , KCl and H_2SO_4 on the resulting current signal. Corresponding cyclic voltammograms recorded in test solution containing 1 mM HCF are presented in Fig. 4, which clearly demonstrates that the most suitable electrolyte yielding the highest current signal and the lowest peak separation of 60 mV between reduction and oxidation peaks for HCF is KCl (thick line). Thus, we used KCl in all subsequent experiments as the most favorable electrolyte for the preconditioning step. The two most important processes involved during the preconditioning step are (i) oxidation of the Carbon Black surface, i.e. its electrochemical modification with carbonyl and carboxyl groups and (ii) partial oxidation of binder oil to enhance electrical cross-communication between carbon nano-particles. The proper selection of the electrolyte has evidently a profound effect upon the preconditioning of the electrode surface. Its role might be associated with different interfacial catalytic reactions between electrolyte ions and the electrode surface.

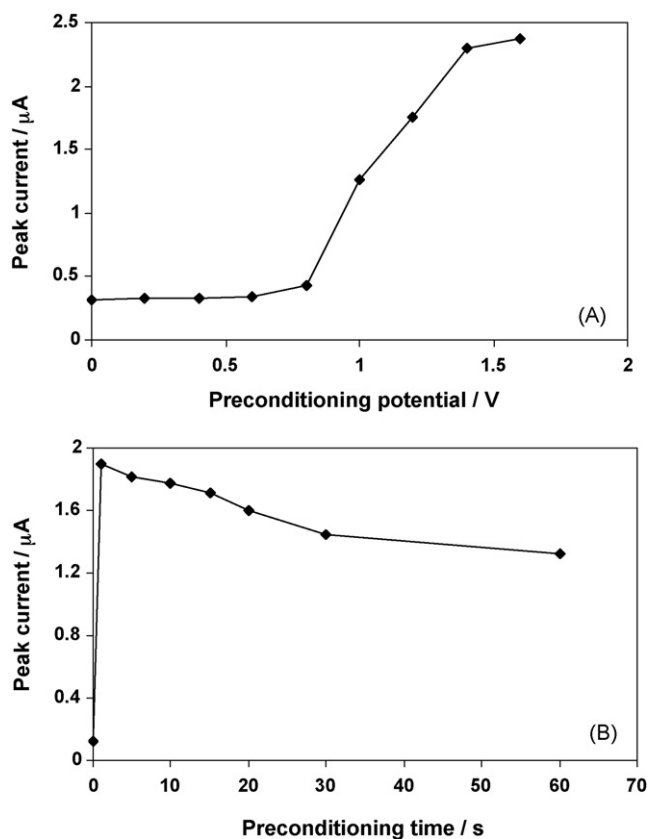


Fig. 3. Effect of preconditioning potential (A) and preconditioning time (B) upon the peak current obtained at carbon paste electrode with radius of 0.5 mm in the solution of 1 mM HCF containing 0.1 M KCl . Other conditions as in Fig. 2.

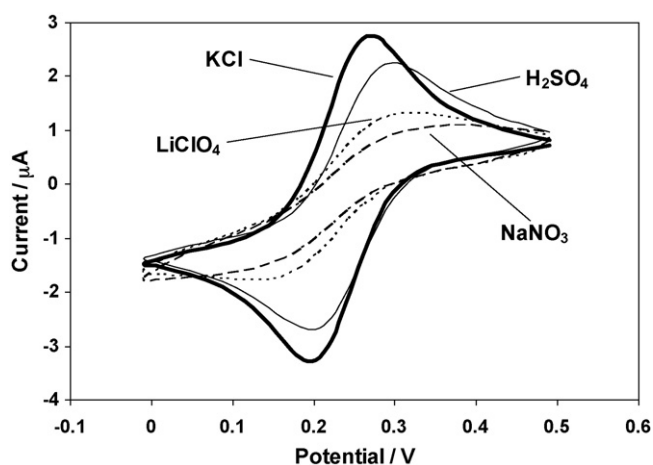


Fig. 4. Cyclic voltammograms obtained at carbon paste electrode with radius of 0.5 mm in the solution of 1 mM HCF containing 0.1 M KCl after preconditioning for 3 s at +1.4 V in the solution of 0.1 M KCl (thick line), 0.1 M H_2SO_4 (thin line), 0.1 M LiClO_4 (dotted line) and 0.1 M NaNO_3 (dashed line). Other conditions as in Fig. 2.

The following experiments were performed using our newly designed piston-driven system in combination with carbon paste micro-electrodes. To get more insights into the electrochemical characteristics of the aforementioned micro-electrodes we studied their response in the solution containing 1 mM HCF and 0.1 KCl with respect to their radius (Fig. 5). In addition, we compared the experimental results with theoretical values, as shown in the inset of Fig. 5, that were calculated using a corrected equation for limiting currents corresponding to disk micro-electrodes embedded in a thin insulation layer [21,25]:

$$I_L = \left(1 + 0.379 \left(\frac{r}{r+d} \right)^{2.342} \right) 4nFD Cr,$$

where I_L stands for limiting current, r for radius of the CPME, d for thickness of the insulating layer, n for number of involved electrons during electrochemical redox reaction, F for Faraday constant, D for diffusion coefficient of electroactive species, i.e. HCF ($7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$) and C for its bulk concentration. Evidently, the experimental values ((a) in the inset of Fig. 5) match perfectly with theoretical ones ((b) in the inset of Fig. 5) for radius of 4.5, 14 and 22 μm , while the CPME with radius of 62 μm exhibited lower signal than that expected with accordance to the theory. Note also the translation from sigmoidal-shaped cyclic voltammograms to peak-shaped cyclic voltammogram in the case of electrode with radius of 62 μm due to prevailing planar diffusion over radial diffusion. The discrepancy concern-

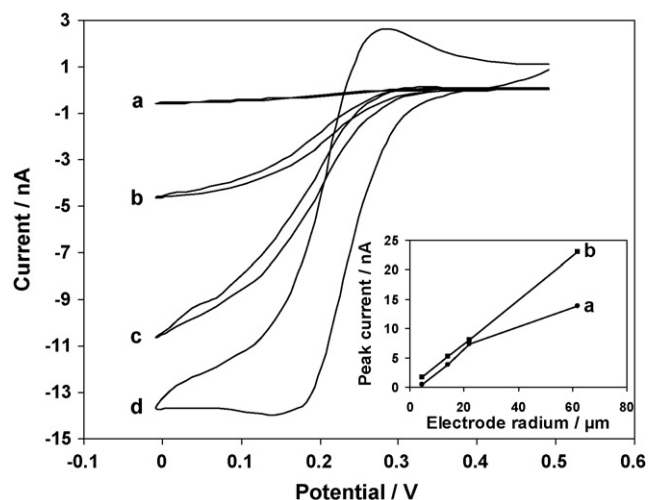


Fig. 5. Cyclic voltammograms obtained at carbon paste micro-electrodes with radius of 4.5 μm (a), 14 μm (b), 22 μm (c) and 62 μm (d) in the solution of 1 mM HCF containing 0.1 M KCl. Inset shows the corresponding plots of cathodic peak current vs. electrode radius for experimental (a) and calculated (b) current values. Other conditions as in Fig. 2.

ing lower current signal might have origins also in lower current density as a result of different diffusion profiles, and on the other hand, in larger electrode surface resulting in larger currents and consequently higher Ohmic drop due to internal resistance of the whole examined system. However, with these experiments

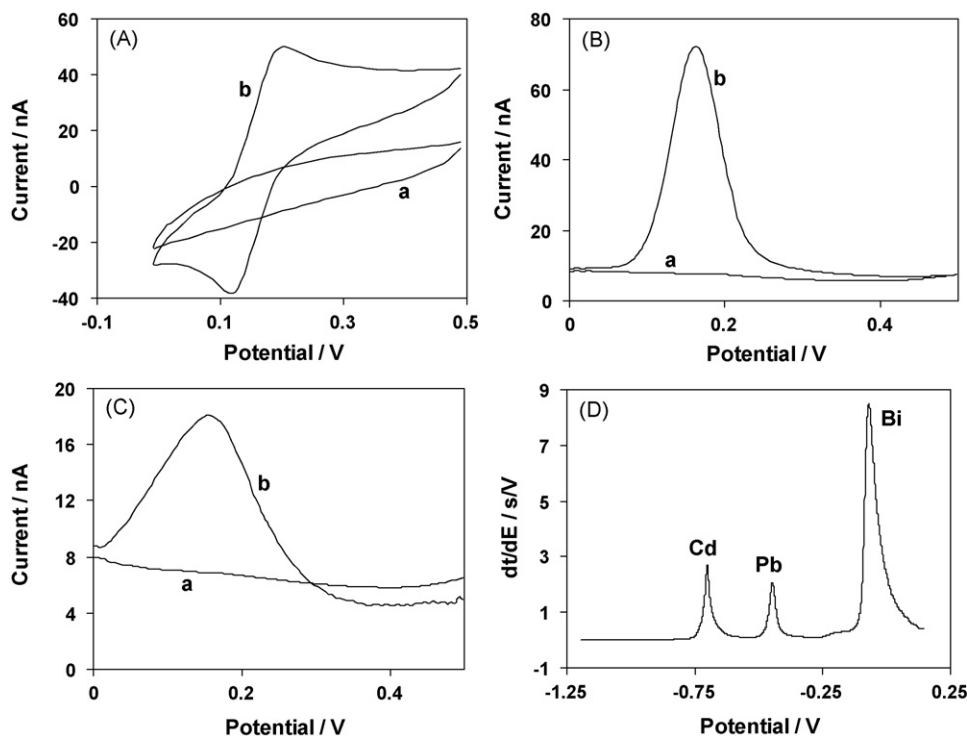


Fig. 6. Cyclic voltammogram for 10 μM dopamine (A), square-wave voltammogram for 10 μM dopamine (B), square-wave voltammogram for 10 μM ascorbate (C), and stripping chronopotentiogram for 80 $\mu\text{g L}^{-1}$ cadmium(II) and lead(II) obtained at CPME ($r = 15 \mu\text{m}$). Solutions, 0.1 M phosphate buffer solution (A, B, C); 0.05 M acetate buffer solution containing 400 $\mu\text{g L}^{-1}$ of Bi(III) (D). Operational parameters, cyclic voltammogram (A): scan rate = 100 mV s^{-1} ; square-wave voltammograms (B and C): frequency = 10 Hz, potential step = 5 mV and amplitude = 25 mV; stripping chronopotentiogram (D): deposition potential = -1.2 V , stripping current = 0.05 μA , equilibration time = 15 s.

we undisputedly shown that the newly developed CPME possesses auspicious electrochemical characteristics enabling its wider applicability in electroanalysis, also in combination with its bulk or surface modifications.

To get further insights into its electroanalytical performance, the CPME with radius of 15 μm was further employed for voltammetric measurements of some selected potentially interesting compounds such as dopamine and ascorbic acid, in this preliminary stage at the unmodified electrode surface, and in addition some heavy metals in combination with in situ prepared bismuth film carbon paste micro-electrode using stripping chronopotentiometry. In Fig. 6 we can see several applications in combination with different electrochemical detection modes, i.e. voltammetric operation of CPME for convenient measurement of dopamine (A, B) and ascorbic acid (C) and stripping chronopotentiometric detection of $\mu\text{g L}^{-1}$ concentration levels of cadmium(II) and lead(II) (D). A well-defined and relatively high cyclic voltammetric signal for 10 $\mu\text{mol L}^{-1}$ of dopamine is clearly developed (Fig. 6Ab) over the flat background voltammogram (Fig. 6Aa). As characteristic for dopamine, both oxidation and reduction signals can be observed with a peak separation of approximately 85 mV unveiling nearly reversible redox behavior. Fig. 6B also depicts the operation of CPME in a square-wave voltammetric mode exhibiting undistorted and sharp signal for the same concentration of dopamine at +0.16 V (6Bb) together with low background contribution (6Ba). Similar square-wave voltammetric experiments were conducted in the case of 10 $\mu\text{mol L}^{-1}$ ascorbate with a peak potential corresponding to the oxidation of ascorbate, similar as dopamine, at approximately +0.16 V (6Cb). The signal for ascorbate is significantly broader compared with that corresponding to dopamine in Fig. 6B reflecting the irreversible character of the oxidation process. As expected, in cyclic voltammetric mode we did not observe any signal except the background current during the backward scan, also consistent with the irreversible oxidation process of ascorbate (not shown). Due to the early stage of this work, we did not perform detailed studies concerning the limit of detection, calibration, etc., however we continued our investigation to prove the suitability of our new micro-electrode for measuring trace heavy metals using chronopotentiometric stripping analysis at the in situ prepared bismuth film CPME. As a model solution, we prepare a 0.05 M acetate buffer solution containing 80 $\mu\text{g L}^{-1}$ of both cadmium(II) and lead(II) together with 400 $\mu\text{g L}^{-1}$ of bismuth(III). As evident from Fig. 6D, the CPME exhibited attractive electroanalytical performance with well-separated stripping signals (ca. 0.26 V) for both metal analytes. The stripping chronopotentiogram did not reflect any adverse effects due to the presence of dissolved oxygen in the measurement solution and in addition, it revealed undistorted signals indicating satisfactory coverage of the Carbon Black-based electrode surface with thin bismuth film.

A simple and reliable piston-driven micro-electrode holder together with favorable electroanalytical characteristics of carbon paste material encompassing carbon nano-particles hold great promise for broader application of carbon paste-based electrochemical sensors also in more challenging environments,

such as measurements in micro-volumes, at micro-locations, measurements in the samples with low conductivity or where convection cannot be controlled, in vivo measurements, etc.



Happy Birthday!

4. Conclusions

In this work, we have demonstrated the preparation, characterization and applicability of the carbon paste micro-electrode (CPME) which is based on carbon nano-particles with an average diameter of 30 nm (Carbon Black) mixed together with binding oil. The carbon paste electrode material was encased in a pulled glass capillaries with radius down to 4.5 μm and the manipulation of CPMEs was accomplished via newly developed piston-driven micro-electrode holder which at the same time enabled convenient renewal of the electrode surface. After optimizing the composition of carbon paste and its electrochemical preconditioning, the CPMEs exhibited excellent electroanalytical performance for measuring some potentially interesting organic and inorganic analytes, such as dopamine, ascorbate and selected heavy metals, i.e. cadmium(II) and lead(II). Combining the attractive characteristics of highly conductive carbon nano-particles with unique electrochemical behavior of micro-electrodes expands the scope and applicability of carbon paste-based electrodes for measurements in new and more challenging environments.

Acknowledgements

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References

- [1] R.N. Adams, *Anal. Chem.* 30 (1958) 1576.
- [2] K. Kalcher, J. Wang, J.-M. Kauffmann, I. Svancara, K. Vytras, C. Neuhold, Z. Yang, *Electroanalysis* 7 (1995) 5.
- [3] S.B. Hočevár, I. Svancara, K. Vytras, B. Ogorevc, *Electrochim. Acta* 51 (2005) 706.
- [4] G.L. Luque, M.C. Rodríguez, G.A. Rivas, *Talanta* 66 (2005) 467.
- [5] P. Kotzian, P. Brázdilová, K. Kalcher, K. Handlíř, K. Vytras, *Sens. Actuator B* 124 (2007) 297.
- [6] J. Wang, L. Chen, S.B. Hočevár, B. Ogorevc, *Analyst* 125 (2000) 1431.
- [7] G.-U. Flechsig, M. Kienbaum, P. Gründler, *Electrochem. Commun.* 7 (2005) 1091.
- [8] E.V. Ivanova, V.S. Sergeeva, J. Oni, C. Kurzawa, A.D. Ryabov, W. Schuhmann, *Bioelectrochemistry* 60 (2003) 65.

- [9] C. Nistor, J. Emnéus, L. Gorton, A. Ciucu, *Anal. Chim. Acta* 387 (1999) 309.
- [10] J. Zheng, X. Zhou, *Bioelectrochemistry* 70 (2007) 408.
- [11] S. Alpat, S.K. Alpat, A. Telefoncu, *Anal. Bioanal. Chem.* 383 (2005) 695.
- [12] J. Wang, X. Zhang, M. Prakash, *Anal. Chim. Acta* 395 (1999) 11.
- [13] J. Oni, P. Westbroek, T. Nyokong, *Electrochem. Commun.* 3 (2001) 524.
- [14] Y. Zou, J. Mo, *Anal. Chim. Acta* 382 (1999) 145.
- [15] J. Calvo Quintana, L. Idrissi, G. Palleschi, P. Albertano, A. Amine, M. El Rhazi, D. Moscone, *Talanta* 63 (2004) 567.
- [16] J. Wang, R.P. Deo, P. Poulin, M. Mangey, *J. Am. Chem. Soc.* 125 (2003) 14706.
- [17] S. Timur, U. Anik, D. Odaci, L. Gorton, *Electrochem. Commun.* 9 (2007) 1810.
- [18] M.D. Rubianes, G.A. Rivas, *Electrochem. Commun.* 5 (2003) 689.
- [19] M. Pumera, A. Merkoçi, S. Alegret, *Sens. Actuator B* 113 (2006) 617.
- [20] C.G. Zoski, *Electroanalysis* 14 (2002) 1041.
- [21] X. Zhang, B. Ogorevc, *Anal. Chem.* 70 (1998) 1646.
- [22] J.W. Mo, B. Ogorevc, *Anal. Chem.* 73 (2001) 1196.
- [23] S.B. Hocevar, M. Zivin, A. Milutinovic, M. Hawlina, E.A. Hutton, B. Ogorevc, *Front. Biosci.* 11 (2006) 2782.
- [24] R.H. Baughman, A. Zakhidov, W.A. de Heer, *Science* 297 (2002) 787.
- [25] G. Zhao, D.M. Giolando, J.R. Kirchhoff, *Anal. Chem.* 67 (1995) 2592.

Biomolecules-carbon nanotubes doped conducting polymer nanocomposites and their sensor application

Maxwell C. Kum¹, Kanchan A. Joshi¹, Wilfred Chen,
Nosang V. Myung, Ashok Mulchandani^{*}

*Department of Chemical and Environmental Engineering, and Center for Nanoscale Science and Engineering,
University of California, Riverside, CA 92521, United States*

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Abstract

A simple method for preparing bio-functionalized soluble single-walled carbon nanotubes (SWNTs) is described. Different proteins such as bovine serum albumin (BSA), cytochrome *c* and horseradish peroxidase (HRP) were used to solubilize low functionality SWNTs in water aided by sonication. The unbound proteins were removed by column chromatography and the SWNT-protein conjugate was used as the sole anionic dopant in electropolymerization of polypyrrole from polymerization solution at pH above the isoelectric point of the protein to provide a negative charge. The morphology of the polypyrrole with SWNT-protein dopant was found to be three-dimensional and fibrous with wide open interlocking pores in contrast to smooth and cauliflower-like for chloride doped polypyrrole. Enhanced sensor performance was demonstrated for hydrogen peroxide detection on polypyrrole/SWCNT-HRP nanocomposites modified electrode. Such nanocomposites can be potentially applied for other biosensor and bio-fuel cell applications.

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Keywords: Carbon nanotubes; Biomolecule; Conducting polymer; Polypyrrole; Nanocomposite; Biosensor

1. Introduction

Carbon nanotubes (CNTs) exhibit unique electronic, metallic, and structural characteristics [1]. Due to these interesting properties there are reports in literature about the sensor applications of CNTs [2,3]. CNTs show electrocatalytic activity toward biologically important compounds such as dopamine, epinephrine, and ascorbic acid [4], NADH [5], phenolic compounds [6], hydrogen peroxide [7], and thiol compounds such as cysteine, glutathione, and thiolcholine [8,9]. CNTs also possess high surface area and have been used to facilitate immobilization of biological molecules [10–12] and for biosensor applications [13–15].

Another important class of organic material for electronic devices is conducting polymers (CPs). Conducting polymers contain alternating single and double bonds between the car-

bon atoms in their backbone which provide tunable electronic properties (metallic to insulator), low energy optical transitions, low ionization potential and high electron affinity [16–20]. However, most of these properties depend on the synthesis procedure as well as on the dopant nature [21]. Furthermore, conducting polymers exhibit poor mechanical strength. To this end, CNTs doped CP nanocomposites have been explored to improve the mechanical and electrical properties of CPs [22–27].

The use of CNTs for promising applications of biosensors, bio-fuel cells, etc., is made difficult due to their poor solubility in most solvents [28,29]. Of the several methods reported for the solubilization of CNTs, non-covalent functionalization is promising because of the simplicity and as it does not affect the structure of CNTs [30]. In the present work, we report for the first time a simple and mild method to bio-functionalize and solubilize single-walled CNTs (SWNTs) in a single step based on non-covalent adsorption of proteins on to SWNT and the incorporation of the SWNT-protein conjugates in polypyrrole as sole anionic dopant to make nanocomposites for biosensing applications. This unique method has potential for other important applications such as bio-fuel cells.

^{*} Corresponding author. Tel.: +1 951 827 6419; fax: +1 951 827 6419.

E-mail address: adani@engr.ucr.edu (A. Mulchandani).

¹ These authors have contributed equally to the work.

2. Experimental

2.1. Materials

Single-walled carbon nanotubes with low functionality, sold under the trade name of P2-SWNT, were provided by Carbon Solutions, Inc. (Riverside, CA, USA). Bovine serum albumin (BSA), horseradish peroxidase (HRP), cytochrome *c* (Cyt_c), and pyrrole monomer were purchased from Sigma–Aldrich (Milwaukee, WI) and all other chemicals and supplies were purchased from Fisher Scientific (Tustin, CA, USA).

2.2. Solubilization/dispersion of SWNTs with proteins and purification of SWCNT-protein conjugate

One milligram per milliliter of SWNTs and 5 mg/ml of protein (i.e. BSA, HRP, or Cyt_c) were dissolved in deionized H₂O. The mixture was sonicated for 20 min and then centrifuged for 5 min at 13800 × *g*.

Gel chromatography was used to purify the SWNT-protein conjugate from excess protein. Sephadex G-150 that was pre-soaked and deaerated using a vacuum pump was packed up to 16 cm in a 2.5 cm diameter × 22 cm long glass column. The SWNT-protein supernatant recovered after centrifugation was layered on the top of the gel and eluted using water flowing under gravity. Volume fractions were collected for 1 min duration using a Bio-Rad model 2110 fraction collector (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed for the presence of SWNTs by measuring absorbance at 500 nm using spectrophotometer (Cary 1E, Varian Inc., Melbourne, Australia) and protein by the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and/or absorbance at 280 nm for BSA and 405 nm corresponding to heme for HRP and cytochrome *c*. Fractions showing high absorbance at 725 nm and protein content were pooled for further use.

2.3. Characterization of SWNT-protein conjugate and PPy/SWNT-protein nanocomposite

Scanning electron microscopy (Leo SUPRA 55, Carl Zeiss, Germany) and atomic force microscopy (Nanoscope E and associated equipments, Veeco Instruments, Inc. San Clemente, CA, USA) were used for investigation of the surface morphology.

Vis–NIR absorption spectra for the SWNT-protein conjugate were measured using Mikropack DH-2000 UV–Vis–NIR Light Source and Ocean Optics HR4000 High Resolution Spectrometer (Ocean Optics Inc., Dunedin, FL, USA) using a quartz cuvette.

2.4. Electropolymerization and H₂O₂ detection

Pyrrole was added to the SWNT-protein conjugate pooled fractions for a final concentration of 0.5 M. The mixture was sonicated for 15 min, the pH adjusted to 7 and then purged with nitrogen gas for 10 min for deaeration.

The electropolymerization was performed using cyclic voltammetry (CV) from –0.65 to 1.05 V for 50 cycles using a

multi potentio/galvanostat (VMP2, Princeton Applied Research, Oak Ridge, TN, USA). Three electrode configurations were used for electropolymerization where a polished glassy carbon electrode, platinum wire and Ag/AgCl in 3 M KCl were used as working, counter and reference electrode, respectively. The scan rate was fixed at 50 mV/s.

After the electropolymerization, the PPy/SWNT-protein thin film was subjected to overoxidation by cycling the potential from –0.2 to 1.3 V for 50 cycles at 100 mV/s in a deaerated phosphate buffer solution. The overoxidized film was tested for response to 5×10^{-3} M hydrogen peroxide at varying potential from –0.3 to 0.2 V vs. Ag/AgCl reference electrode using a BASi Amperometric Detector (LC-4C, BASi Inc., West Lafayette, IN, USA).

3. Results and discussion

3.1. Solubilization/dispersion of SWNTs with proteins

In an aqueous environment, pristine SWNTs aggregated and settled down at the bottom of the vial (Fig. 1, left), but with the addition of BSA, the SWNTs appeared well dispersed in a suspension (Fig. 1, right). This SWNT-BSA conjugate was stable for well over 2 weeks at room temperature. It has been demonstrated that nonspecific binding occurs between the hydrophobic surface region of a protein and the side wall of a SWNT [31]. With proteins adsorbed on the surface of the SWNT, the SWNT-protein became more water soluble as the surface of the nanostructures became more hydrophilic. This is in agreement with other published work [32]. HRP also displayed similar results whereas Cyt_c was visually inconclusive and would be verified in a subsequent analytical experiment. The solubilization of SWNT by protein adsorption opens a facile and mild route

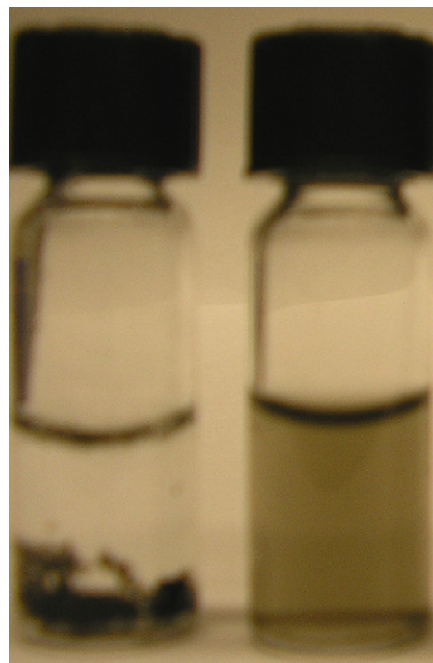


Fig. 1. Photograph of pristine SWNTs (left vial) and SWNT-BSA (right vial) in an aqueous environment after 2 weeks.

for synthesizing functional SWNT-protein conjugate in comparison to the covalent attachment routes demonstrated to-date. The elimination of harsh chemicals and conditions employed in functionalization of CNTs with biomolecules are the added advantages of the present methodology.

3.2. Characterization of SWNT-protein conjugates

The morphology of SWNT-BSA conjugate was characterized with SEM and AFM. The SEM images (Fig. 2) distinctly differentiated between the highly dispersed state of SWNT-BSA conjugates and the aggregated state of pristine SWNTs. The AFM image (Fig. 3) of a single SWNT-BSA conjugate structure showed numerous globular structures binding to a linear structure potentially consisting of multiple SWNTs. The length and diameter of the structure in the AFM image suggested that there were multiple SWNTs since the dimensions of the entire structure were well over the typical length and diameter of a single SWNT. It was very likely that the hydrophobic side walls of several SWNTs bound to each other in a stacking manner as well as to the hydrophobic surface of the proteins. The size of the globular structures from the AFM image also indicated numerous BSA proteins amassing together. These images confirmed the formation of SWNT-protein conjugate.

The amounts of CNTs dispersed can be determined from the absorbance in either the visible range at 500 nm or in the near-

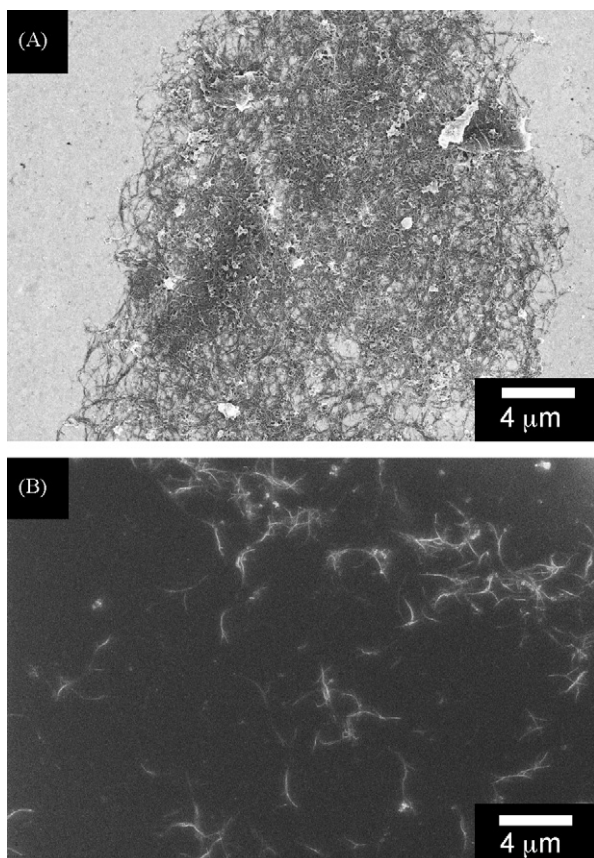


Fig. 2. SEM images of pristine SWNTs (A) and SWNT-BSA conjugates (B).

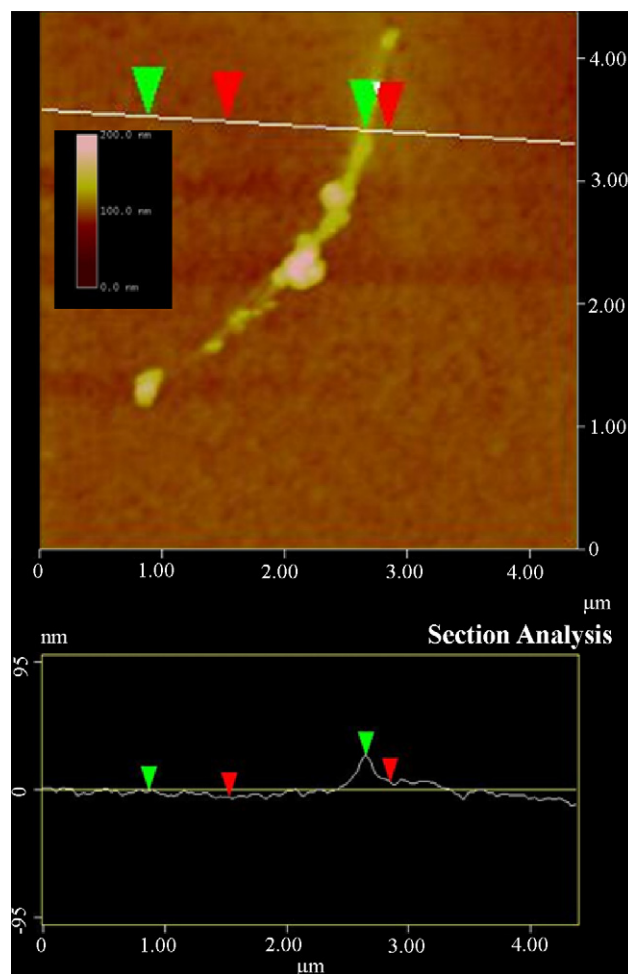


Fig. 3. AFM image of SWCNT-protein conjugate.

infra red region at 1025 nm [33,34]. While the absorbance in the visible range has no specific attribute to any CNT property, the NIR absorbance is a characteristic of the SWNTs relating to the S₂₂ interband transition. The absorbance of the SWNT-protein dispersion at both wavelengths indicated the presence of SWNTs in the dispersion and the trend of decreasing solubility in the order of BSA > HRP > cytochrome *c*. Based on 1025 nm absorbance, for which there is no reported extinction coefficient, the SWNT-BSA was approximately five times more soluble than SWNT-HRP, whereas SWNT-cytochrome *c* was very weakly soluble while solubility calculated based on the reported extinction coefficient of 28.6 L g⁻¹ cm⁻¹ for HiPco SWNT [35] at 500 nm the solubility were 52.4, 21.3 and 3.8 mg/L for SWNT-BSA, SWNT-HRP and SWNT-Cytc, respectively (Fig. 4). The drastic difference in solubility can be attributed to a varying level of hydrophobic interaction between the proteins and the SWNTs resulting in a varying amount of solubilized SWNTs. If a protein does not have a sufficiently large surface area of hydrophobic groove on its surface, then the level of hydrophobic interaction with SWNTs can be significantly reduced and thus negatively affect the solubilization via non-covalent functionalization. The predominant absorbance peak at 405 nm for HRP and cytochrome *c* is attributed to the heme

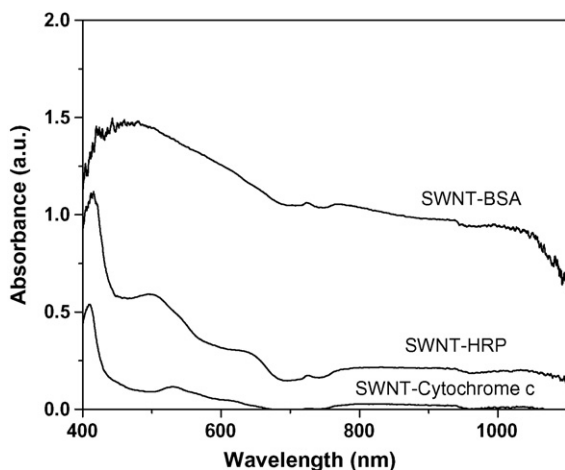


Fig. 4. Vis-NIR absorption spectra of SWNT-proteins.

in these proteins and is an additional confirmation of the presence of these proteins in the conjugates and the concentration trend.

3.3. Electropolymerization of PPy with SWNT-protein conjugate as dopant

CNTs solubilized by introducing -COOH groups or by covalent attachment of poly(*m*-aminobenzene sulfonic acid) have been used as a dopant in polypyrrole film to improve the conductivity and mechanical strength of the film [27,36]. In this work, SWNT solubilized through protein adsorption was applied as the dopant instead. Since the *pI* of BSA is around 4.7 and the *pI* of the 7 isozymes of HRP is reported to be between 3 and 9, both SWNT-proteins conjugates can have overall net negative charges at the pH 7 used for electrochemical polymerization which would allow SWNT-protein conjugates to act as dopants in a polypyrrole film. Pyrrole was added to the purified SWNT-protein suspension and electropolymerized into a film using cyclic voltammetry (Fig. 5). The CVs exhibited typical growth behaviors of an electropolymerization process with a pronounced reduction peak. Because no electrolyte has been added into the deposition bath, SWNT-protein was acting as both an electrolyte and a dopant during the electropolymerization process. The current was much lower during the growth in comparison to the PPy/ Cl^- film because of the higher conductivity and concentration of Cl^- anion compared to the SWNT-protein conjugate. SWNT-cytochrome *c* was not used in this experiment because of the very low concentration. For SWNT-cytochrome *c* conjugate to act as dopant, the electropolymerization would have to be performed at pH above 11 because the *pI* of cytochrome *c* is in the range of 10.37–10.80.

As shown in Fig. 6, the surface morphology of the PPy films differed remarkably between the PPy/SWNT-BSA and PPy/ Cl^- films. SEM image of PPy/SWNT-BSA film revealed a very fibrous three-dimensional reticular structure with interlocking pores whereas the image of PPy/ Cl^- displayed a typical smooth and cauliflower morphology. As the solubilized SWNT-BSAs were being integrated into the polypyrrole film during the elec-

tropolymerization process, polypyrrole also coated the surfaces of these conjugates and produced these fibrous structures on the film. These images further verified that SWNT-BSA have been embedded in the polymer matrix as a dopant. The fibrous nature of the film allows the film to be more porous with increased surface area, which would reduce the mass transfer limitation compared to a thick polypyrrole film. Having a larger surface area and lower mass transfer limitation facilitating easier access

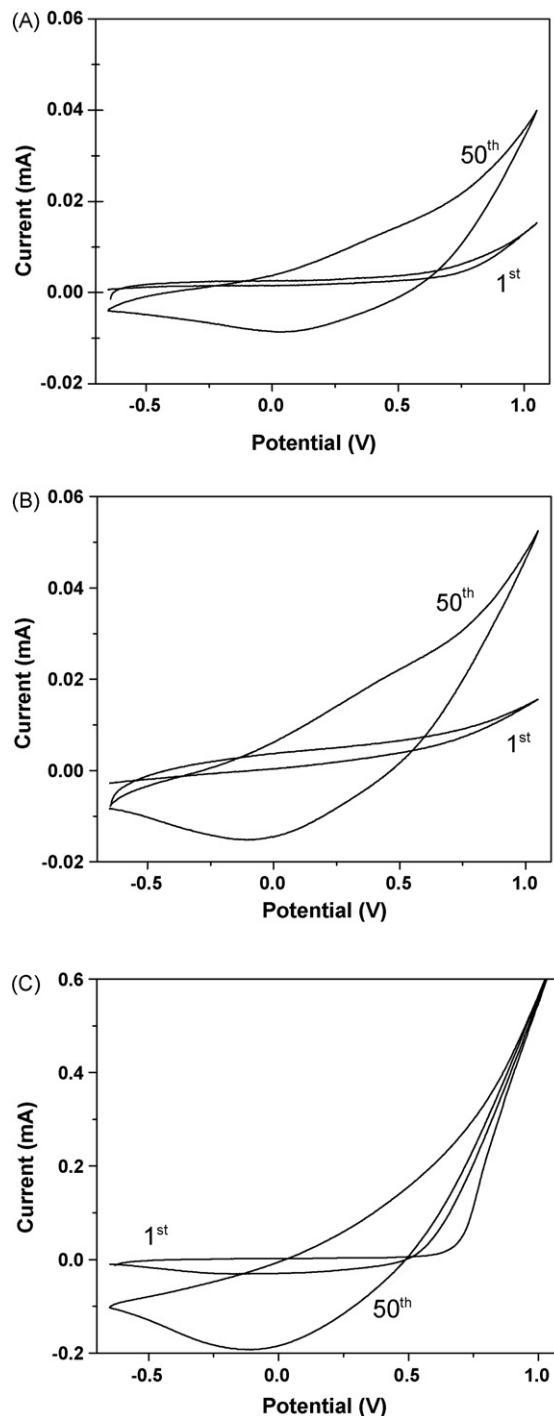


Fig. 5. Potentiodynamic electropolymerization of PPy with (A) SWNT-BSA, (B) SWNT-HRP, and (C) Cl^- as dopants. Pyrrole concentration was fixed at 0.5 M. The scan rate was at 50 mV/s for 50 cycles.

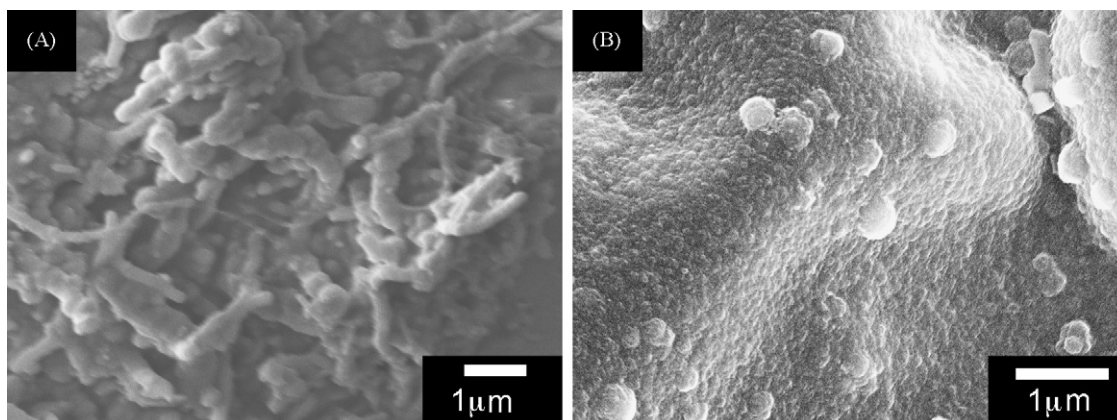


Fig. 6. SEM images of (A) PPy/SWNT-BSA and (B) PPy/Cl[−] thin films.

to substrate are favorable for many engineering applications such as fuel cells, in addition to the sensor application demonstrated in the current work.

3.4. Application of SWNT-HRP conjugate doped polypyrrole composite

Recent studies have shown that CNTs can enhance electrochemical reactivity of biomolecules and promote electron transfer reactions of proteins. To evaluate the potential of SWNT-protein doped polypyrrole film for biosensor, we studied the response to H₂O₂ of PPy/SWNT-HRP nanocomposites film and compared to the response of the PPy film in which the HRP (no SWNT) was entrapped during the electropolymerization with chloride dopant by performing hydrodynamic voltammetry (HDV). Since PPy is conducting and also has reducing power and can interfere in the signal from the interaction of the SWNT-HRP or HRP with hydrogen peroxide, the films were overoxidized to render them non-conducting/insulating. As shown in Fig. 7, the CVs for both films at the end of the overoxidation appeared identical in phosphate buffer solution transforming the polypyrrole matrix insensitive to H₂O₂ and thereby allowing the measured signal to be originated from the reduction of H₂O₂ by SWNT-HRP or HRP only.

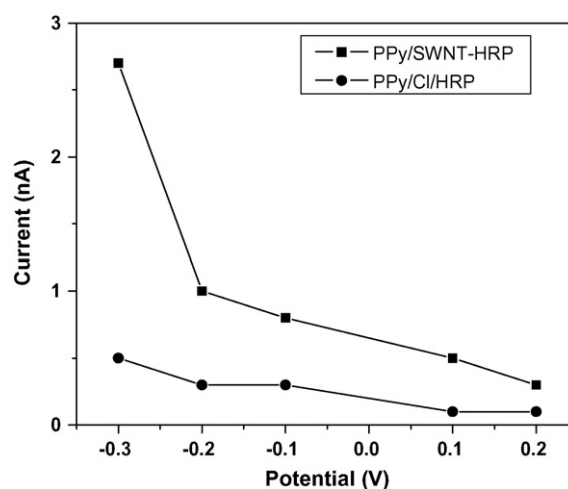


Fig. 8. Hydrodynamic voltammogram of PPy/SWNT-HRP and PPy/Cl[−]/HRP films with 5 mM H₂O₂ as the analyte.

Fig. 8 shows a comparison of the response of PPy/SWNT-HRP and PPy/Cl[−]/HRP modified electrodes as a function of applied potential to 5 mM H₂O₂. The output current over the full range of potential for the SWNT-HRP doped PPy was higher than for the HRP modified chloride doped PPy. The results show specifically at −0.3 V, the improvement in response was well

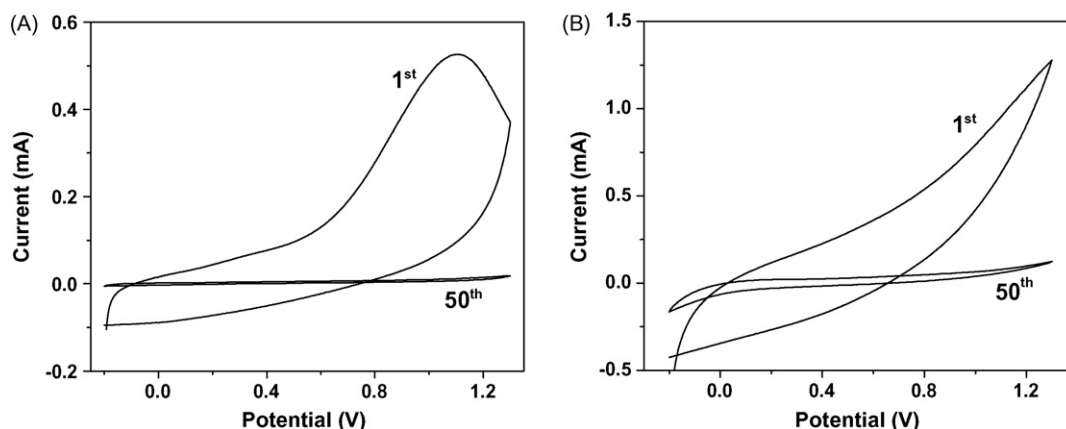


Fig. 7. Overoxidation of (A) PPy/SWNT-HRP and (B) PPy/Cl[−]/HRP films.

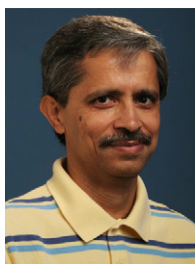
over 5-fold in comparison against the PPy/Cl/HRP film. The enhanced response can be attributed to several factors. First, the enhanced electron transfer within the polypyrrole matrix from the presence of SWNTs. Second, the direct electron transfer from the active site of the enzymes to the electrode through the SWNT bridging them. Third, improved accessibility of the enzyme catalytic sites for the substrate due to highly open reticular morphology of the nanocomposite film. Lastly, the increased loading of the HRP in the film resulting from the incorporation of SWNT-protein as a dopant and improved efficiency of protein immobilization by adsorption on the SWNTs over the conventional method of entrapment in the film.

4. Conclusion

We demonstrated for the first time a simple and benign method for one-step solubilization and functionalization of CNTs through adsorption under mild/benign conditions and the application of the SWCNT-protein conjugates as dopants for the facile synthesis of SWNT-protein-polypyrrole nanocomposites by electrochemical polymerization. The resulting nanocomposites demonstrated enhanced sensor performance when compared to film with entrapped enzyme. Such strategy potentially can be employed for other biosensors and bio-fuel cell applications by simply substituting another protein.

Congratulation/commemoration

Dear Joe, Here's wishing you happiness and many wonderful moments! Cheers to you! Have a very happy 60th birthday!



Acknowledgements

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References

- [1] R.H. Baughman, A.A. Zakhidov, W.A. de Heer, *Science* 297 (2002) 787–792.
- [2] N. Li, J. Wang, M. Li, *Rev. Anal. Chem.* 22 (2003) 19–33.
- [3] B.S. Sherigara, W. Kutner, F. D'Souza, *Electroanalysis* 15 (2003) 753–772.
- [4] H. Luo, Z. Shi, N. Li, Z. Gu, Q. Zhuang, *Anal. Chem.* 73 (2001) 915–920.
- [5] M. Musameh, J. Wang, A. Merkoci, Y. Lin, *Electrochem. Commun.* 4 (2002) 743–746.
- [6] J. Wang, R.P. Deo, M. Musameh, *Electroanalysis* 15 (2003) 1830–1834.
- [7] J. Wang, M. Musameh, Y. Lin, *J. Am. Chem. Soc.* 125 (2003) 2408–2409.
- [8] X.N. Cao, L. Lin, Y.Z. Xian, W. Zhang, Y.F. Xie, L.T. Jin, *Electroanalysis* 15 (2003) 892–897.
- [9] K.A. Joshi, J. Tang, R. Haddon, J. Wang, W. Chen, A. Mulchandani, *Electroanalysis* 17 (2005) 54–58.
- [10] R.J. Chen, Y. Zhang, D. Wang, H. Dai, *J. Am. Chem. Soc.* 123 (2001) 3838–3839.
- [11] J.J. Davis, M.L.H. Green, H.A.O. Hill, Y.C. Leung, P.J. Sadler, J. Sloan, A.V. Xavier, S.C. Tsang, *Inorg. Chim. Acta* 272 (1998) 261–266.
- [12] F. Balavoine, P. Schultz, C. Richard, V. Mallouh, T.W. Ebbesen, C. Mioskowski, *Angew. Chem., Int. Ed.* 38 (1999) 1912–1915.
- [13] Y. Lin, F. Lu, J. Wang, *Electroanalysis* 16 (2004) 145–149.
- [14] A. Guiseppi-Elie, C. Lei, R.H. Baughman, *Nanotechnology* 13 (2002) 559–564.
- [15] R.P. Deo, J. Wang, I. Block, A. Mulchandani, K.A. Joshi, M. Trojanowicz, F. Scholz, W. Chen, Y. Lin, *Anal. Chim. Acta* 530 (2005) 185–189.
- [16] E. Smela, N. Gadegaard, *Adv. Mater.* 11 (1999) 953–957.
- [17] A.G. MacDiarmid, *Angew. Chem. Int. Ed.* 40 (2001) 2581–2590.
- [18] V. Saxena, B.D. Malhotra, *Curr. Appl. Phys.* 3 (2003) 293–305.
- [19] T.F. Otero, I. Boyano, M.T. Cortés, G. Vázquez, *Electrochim. Acta* 49 (2004) 3719–3726.
- [20] L. Dai, P. Soundarrajan, T. Kim, *Pure Appl. Chem.* 74 (2002) 1753–1772.
- [21] A. Ramanaviciene, A. Ramanavicius, in: D.W. Thomas (Ed.), *Advanced Biomaterials for Medical Applications*, Kluwer Academic Publishers, Netherlands, 2004, p. 111.
- [22] M. Hughes, G.Z. Chen, M.S.P. Shaffer, D.J. Fray, A.H. Windle, *Chem. Mater.* 14 (2002) 1610–1613.
- [23] C. Downs, J. Nugent, P.M. Ajayan, D.J. Duquette, K.S.V. Santhanam, *Adv. Mater.* 11 (1999) 1028–1031.
- [24] M. Gao, S. Huang, L. Dai, G. Wallace, R. Gao, Z. Wang, *Angew. Chem., Int. Ed.* 39 (2000) 3664–3667.
- [25] Z. Wei, M. Wan, T. Lin, L. Dai, *Adv. Mater.* 15 (2003) 136–139.
- [26] H. Zengin, W. Zhou, J. Jin, R. Czerw, D.W. Smith Jr., L. Echegoyen, D.L. Carroll, S.H. Foulger, J. Ballato, *Adv. Mater.* 14 (2002) 1480–1483.
- [27] G.Z. Chen, M.S.P. Shaffer, D. Coleby, G. Dixon, W. Zhou, D.J. Fray, A.H. Windle, *Adv. Mater.* 12 (2000) 522–526.
- [28] A. Star, J.F. Stoddart, D. Steuerman, M. Diehl, A. Boukai, E.W. Wong, X. Yang, S.W. Chung, H. Choi, J.R. Heath, *Angew. Chem., Int. Ed.* 40 (2001) 1721–1725.
- [29] J. Chen, M.A. Hamon, H. Hu, Y. Chen, A.M. Rao, P.C. Eklund, R.C. Haddon, *Science* 282 (1998) 95–98.
- [30] M.J. O'Connell, P. Boul, L.M. Ericson, C. Huffman, Y. Wang, E. Haroz, C. Kuper, J. Tour, K.D. Ausman, R.E. Smalley, *Chem. Phys. Lett.* 342 (2001) 265–271.
- [31] R.J. Chen, S. Bangsaruntip, K.A. Drouvalakis, N.W.S. Kim, M. Shim, Y. Li, W. Kim, P.J. Utz, H. Dai, *Proc. Natl. Acad. Sci. U.S.A.* 100 (2003) 4984–4989.
- [32] S.S. Karajanagi, H. Yang, P. Asuri, E. Sellitto, J.S. Dordick, R.S. Kane, *Langmuir* 22 (2006) 1392–1395.
- [33] B. Zhao, H. Hu, A. Yu, D. Perea, R.C. Haddon, *J. Am. Chem. Soc.* 127 (2005) 8197–8203.
- [34] P. Asuri, S.S. Karajanagi, E. Sellitto, D.-Y. Kim, R.S. Kane, J.S. Dordick, *Biotechnol. Bioeng.* (2006) 804–811.
- [35] J. Bhar, E.T. Mickelson, M.J. Bronikowski, R.E. Smalley, J.M. Tour, *Chem. Commun.* (2001) 93–194.
- [36] A.K. Wanekaya, Y. Lei, E. Bekyarova, W. Chen, R. Haddon, A. Mulchandani, N.V. Myung, *Electroanalysis* 18 (2006) 1047–1054.

Molecular anchoring of anthracene-based copolymers onto carbon nanotubes: Enhanced pH sensing

Nathan S. Lawrence*, Kay L. Robinson

Schlumberger Cambridge Research, High Cross, Madingley Road, Cambridge CB3 0EL, UK

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Abstract

The effect of carbon nanotubes on the electrochemical response of the pH sensing, redox-active copolymer poly(vinylanthracene-co-vinylferrocene) has been studied. A clear increase in the linear response of the anthracene moiety with pH is observed in the presence of carbon nanotubes.

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Keywords: Vinylferrocene; Vinylanthracene; Carbon nanotubes; pH; Voltammetry; Polymer

1. Introduction

Since their discovery in 1991 [1], carbon nanotubes (CNTs) have been studied in a variety of scientific fields. Derivatising CNTs with various molecules [2–6] to target specific analytes provides us with a promising method for their use as analytical sensors. This can be achieved by either direct covalent linkage of the desired molecule, or by means of noncovalent interactions of the molecule with the CNT surface. These noncovalent modifications occur as a result of interactions of π -electron-rich compounds with the highly delocalized π electrons of the CNTs [7].

Noncovalent grafting of polymers onto CNTs can be achieved by two different methods [8]. The first approach is by means of polymer wrapping, which occurs when the monomers are polymerized in the presence of CNTs [9]. This methodology has led to CNTs being derivatised with a host of polymers including poly(pyrrole) [10], poly(aniline) [11], poly(vinyl pyrrolidone), and poly(styrene sulfonate) [12]. With this approach, the polymer has to incur a highly strained conformation; as the polymer wrapping is random, there is no control over the spacing of the polymer's functional groups within the CNT and, therefore, the grafting process can be inefficient. The second approach is that in which polymers associate with CNTs in a nonwrapping fashion

[8]. This occurs by means of π stacking with the CNT, whereby the polymer backbone extends substantially along the length of the CNT.

Following this last approach, we wish to investigate the derivatisation of the CNT with poly(vinylanthracene-co-vinylferrocene) (p(VAn-co-VFc)) (Scheme 1, 1). Recently we reported on the pH [13] and sulfide [14] sensing capabilities of this novel copolymer. This was based on the fact that the redox potential of the anthracene moiety is found to follow a Nernstian variation with pH, whilst the redox potential of the ferrocene moiety is stable with respect to pH (Scheme 1). Furthermore the ferrocene moiety was found to undergo an electrocatalytic reaction with sulfide, thus proffering its use as a dual pH/sulfide sensor [14].

Saveant and Amatore demonstrated that the electrochemical response of anthracene is strongly affected by protonation in aprotic solvents [15,16]. In the following we demonstrate that the strong interaction of the anthracene moiety of the co-polymer with CNT has a profound impact on the ability of the polymer to operate as a pH sensor.

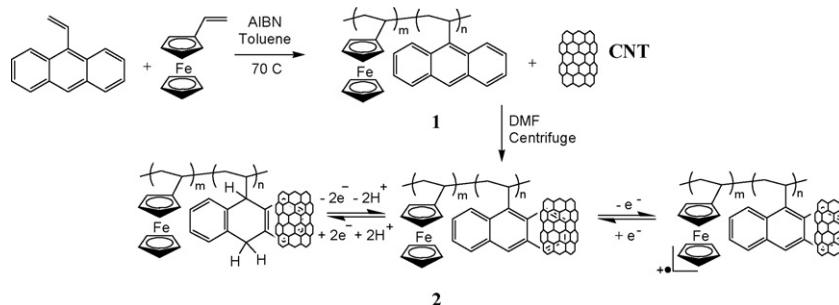
2. Experimental

2.1. Reagents

All chemicals were supplied by Aldrich and used without further purification. The pH 4, 7, and 9 buffered solutions were

* Corresponding author.

E-mail address: nlawrenc@cambridge.oilfield.slb.com (N.S. Lawrence).



Scheme 1. Reaction scheme for the copolymerisation of vinylanthracene and vinylferrocene along with the proposed electrochemical oxidation pathway of the poly(VAn-co-VFc) derivatised carbon nanotubes.

made in accordance with the IUPAC standards [17]. Briefly these were prepared as follows, pH 4 potassium phthalate buffer (0.05 M), pH 7 disodium hydrogen phosphate (0.025 M) and potassium dihydrogen phosphate, (0.025 M) and pH 9 disodium tetraborate (0.05 M). For all other pH values studied a Britton Robinson buffer (0.04 M boric acid, 0.04 M phosphoric Acid, and 0.04 M acetic acid) solution was prepared and the pH adjusted using concentrated NaOH.

The polymers were prepared using commercially available vinylferrocene and vinylanthracene.

2.2. Apparatus

Electrochemical measurements were recorded using an μ Autolab II potentiostat (Ecochemie, Netherlands) with a standard three-electrode configuration. A platinum wire (1 mm diameter, Goodfellow) provided the counter electrode and a saturated calomel electrode (SCE, Radiometer, Copenhagen) acted as the reference. Glassy carbon (3 mm diameter, BAS, UK) acted as the working electrode.

The EDAX spectra were collected using Genesis EDAX X-ray microanalysis. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were carried out by ITS Testing Services (UK) Ltd., using a Mettler Toledo Star instrument.

2.3. Synthesis of polymers

The synthesis of the various polymers was analogous to that discussed previously [13,14]. After synthesis, a ¹H NMR analysis was conducted to ensure that no monomer was present in the resulting copolymer.

DSC analysis of the polymers was conducted by heating the various samples from 30 °C to 300 °C at a rate of 10 °C/min under a 100 mL/min air flow. A similar glass transition temperature was observed for both pVFc and p(Van-co-VFc) at ~180 °C, with onset temperatures of 127.83 °C and 130.95 °C, respec-

tively. No crystallization temperatures or melting temperatures were observed implying that the (co)polymers are completely amorphous.

TGA analysis of two polymer samples was carried out by heating the samples from 30 °C to 500 °C at a rate of 10 °C per min under a 100 mL/min air flow. The results are summarised in Table 1. These show that both PVFc and p(Van_{40%}-co-VFc_{60%}) show two weight loss transitions, and both occurring at higher temperature for p(Van_{40%}-co-VFc_{60%}) than for pVFc. Furthermore, the copolymer degrades less than the homopolymer in this first transition (48.5 wt% for p(Van_{40%}-co-VFc_{60%}) compared to 62.2 wt% for pVFc). The temperature range over which this first transition occurs is a lot narrower for the homopolymer (14 °C for pVFc compared to 126 °C for p(Van_{40%}-co-VFc_{60%})). All this information indicates that the Van is stabilising the pVFc in some way.

The copolymer however, loses a further 16.2 wt% in the second transition compared to just 8.6 wt% for the homopolymer, and this occurs over shorter temperature range. This can be tentatively attributed to the degradation of the polymeric backbone.

2.4. Derivatisation of the CNTs

The CNTs (multiwalled carbon nanotubes; diameter 20–40 nm; length 1–5 mm; NanoLab Inc., Newton, MA, USA) were derivatised with the polymers by chemical adsorption. This was achieved by first dispersing 1 mg of CNT into a 1 mL dimethylformamide (DMF) solution containing 1 mg of the polymer under investigation. This solution was then allowed to mix for a period of 2 h, after which the solution was centrifuged at 6000 rpm for 3 min. This allowed the CNT to accumulate in the lower end of the vessel. The top solution was then removed and the resulting CNT washed thoroughly with DMF. This was done to remove any loosely adsorbed species from the carbon surface. This centrifuge process was then repeated until a clear DMF

Table 1
Summary of the results obtained from the TGA analysis of p(VFc) and p(Van_{40%}-co-VFc_{60%})

	Transition	Onset temperature (°C)	Weight loss (wt%)	Temperature range (°C)
pVFc	First	185.98	62.2	14
	Second	316.67	8.6	43
p(VFc-co-VAn)	First	203.98	48.5	126
	Second	384.75	16.2	20

solution was finally removed, thereby indicating the complete removal of the loosely adsorbed polymer from the CNT. The UV–vis spectra of the first and last washing solutions from the p(Van-co-VFc)-CNT solution revealed that the absorbance peak related to the presence of ferrocene in the solution diminished with subsequent washings.

Tunneling electron microscope (TEM) studies of both bare CNT and p(Van-co-VFc)-CNT were carried out. In both cases, the CNTs were well dispersed, and no evidence of copolymer/CNT agglomerate formation was observed [18].

2.5. Immobilisation onto the glassy carbon electrode

The working electrode was prepared by means of a solvent evaporation method. The copolymer or copolymer-CNT (1 mg/mL) was dissolved or dispersed in DMF, and then a known amount of the solution (20 μ L) was cast onto the working electrode surface and allowed to dry at 60 °C. All electrochemical measurements were conducted at 23 °C unless otherwise stated.

3. Results and discussion

The electrochemical responses of two glassy carbon electrodes, one containing poly(vinylanthracene₄₀%-co-vinylferrocene₆₀%) (p(Van₄₀%-co-VFc₆₀%)) and the second containing CNTs derivatised with the same copolymer (p(Van₄₀%-co-VFc₆₀%)-CNT), were first examined. Fig. 1A details the cyclic voltammetric response (scan rate 50 mV s⁻¹) obtained for the p(Van₄₀%-co-VFc₆₀%)-CNT (dashed line) and for the p(Van₄₀%-co-VFc₆₀%) (solid line) when placed in a pH 4 buffer solution. The data shows that the layer containing CNTs has higher capacitive charging currents. Furthermore, it can be seen that for both layers, two well-defined oxidative (a,b) and reductive (a',b') waves are observed. The redox couple at -0.40 V (a,a') can be attributed to the oxidation of the reduced form of the anthracene moiety within the copolymer (Scheme 1) [13,14]. Analysis of this redox couple for each layer reveals that the p(Van₄₀%-co-VFc₆₀%)-CNT layer has a

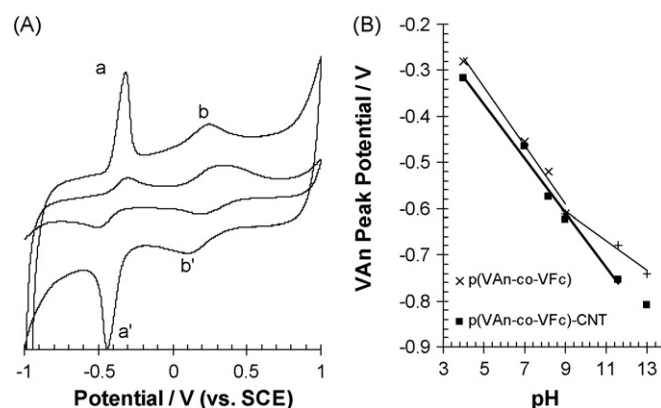


Fig. 1. (A) Cyclic voltammograms (scan rate 50 mV s⁻¹) of both p(Van₄₀%-co-VFc₆₀%) (solid line) and (B) p(Van₄₀%-co-VFc₆₀%)-CNT (dashed line) when placed in a pH 4.0, potassium phthalate (0.05 M) solution. (B) A plot of vinylanthracene peak potential as a function of pH for (vinylanthracene₄₀%-co-vinylferrocene₆₀%) and (vinylanthracene₄₀%-co-vinylferrocene₆₀%) derivatised carbon nanotubes.

lower peak-to-peak separation than the p(Van₄₀%-co-VFc₆₀%) layer, 150 versus 230 mV. This could be tentatively attributed to a higher degree of adsorption of the copolymer wrapped on the carbon nanotubes than that merely dispersed on the planar glassy carbon electrode.

The wave at higher potentials (ca. +0.30 V) can be attributed to the oxidation of the ferrocene moiety of the copolymer chain to the corresponding ferricenium ion [13,14]. It is interesting to note that the oxidation of ferrocene at the p(Van₄₀%-co-VFc₆₀%) layer took place at higher potentials than that at the p(Van₄₀%-co-VFc₆₀%)-CNT layer: +0.35 V versus +0.30 V, respectively. Analysis of the peak current ratios reveals that the anthracene oxidation waves are larger with respect to the ferrocene waves for the p(Van₄₀%-co-VFc₆₀%)-CNT layer compared to those for the p(Van₄₀%-co-VFc₆₀%) layer. The higher π -electron density of anthracene compared to that of ferrocene allows this to preferentially π stack with the CNT surface [19–23].

Fig. 1B depicts a plot of the anthracene peak potential versus pH for the two systems. In the absence of CNTs, two linear

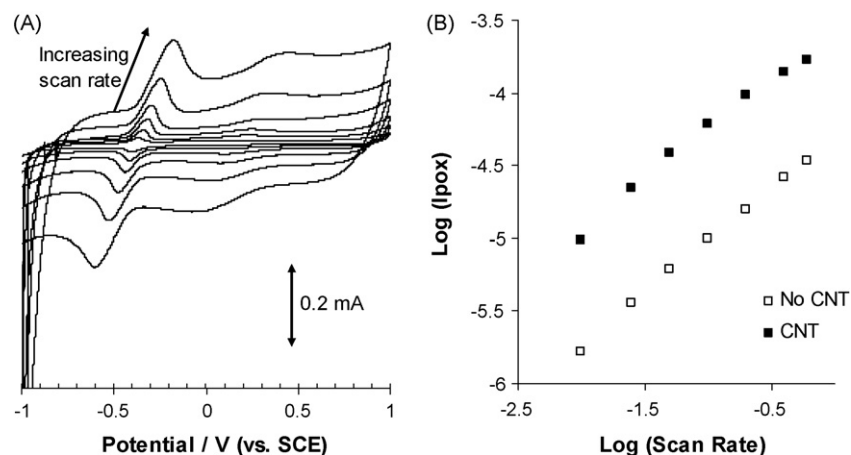


Fig. 2. (A) The effect of scan rate (0.01–0.6 V s⁻¹) on the voltammetric response of p(Van₄₀%-co-VFc₆₀%)-CNT when placed in a pH 4.0, potassium phthalate (0.05 M) buffer solution. (B) Log of peak anthracene peak current vs. log of scan rate for both p(Van₄₀%-co-VFc₆₀%) and p(Van₄₀%-co-VFc₆₀%)-CNT.

regimes are observed: the first, over the pH range of 4.0–9.0, has a slope of 63.1 mV/pH unit, which is consistent with an n -electron to n -proton transfer, where n is likely to be 2 [13,14]. Above a pH 9.0, the anthracene has passed through its pK_a , thereby creating a second regime with a gradient of 31.8 mV/pH unit (2 electrons, 1 proton).

In the presence of CNTs however, the plot of peak potential versus pH is linear up to at least pH 11.6 with a slope of 59.0 mV/pH unit (2 electrons, 2 protons). Above this pH, a deviation from linearity was observed, thus suggesting that the linear response of the anthracene redox potential with pH has increased. For p(Van_{40%}-co-VFc_{60%}) the change in Nernstian slope was observed at pH 9.5, whilst in the presence of CNTs, this occurred at 11.7. This increase can be tentatively attributed to a variety of factors including the possibility of CNTs donating electron density to the anthracene moiety and entropic factors involving solvent ordering/disordering around the interface [24–26].

The voltammetric response of both p(Van_{40%}-co-VFc_{60%}) and p(Van_{40%}-co-VFc_{60%})-CNT was also examined over a wide range of scan rates (10–600 mV s^{−1}). Fig. 2A details the cyclic voltammetric response of p(Van_{40%}-co-VFc_{60%})-CNT at various scan rates. It can be clearly seen that as the scan rate is increased, the magnitude of both redox signals is enhanced as expected. Analysis of the variation of the anthracene peak current as a function of scan rate was carried out using a log(peak current) versus log(scan rate) plot and is shown in Fig. 2B. This shows that larger overall peak currents were observed in the presence of CNTs and is consistent with the data described above. Furthermore, linear responses with gradients of 0.73 in both cases were recorded. This is indicative of a mixed response between the polymers having both diffusion- and surface-bound properties.

To confirm the presence of copolymer upon the CNTs, EDAX spectra of both pure CNT and p(VAn-co-VFc)-CNT were next recorded; the resulting spectra are detailed in Fig. 3A and B, respectively. These reveal that, in the absence of the polymer, the spectrum consists of mainly carbon, oxygen, and silica, which can be attributed to the carbon nanotube layer on the silica substrate. In the case where polymer is assumed to be stacked onto

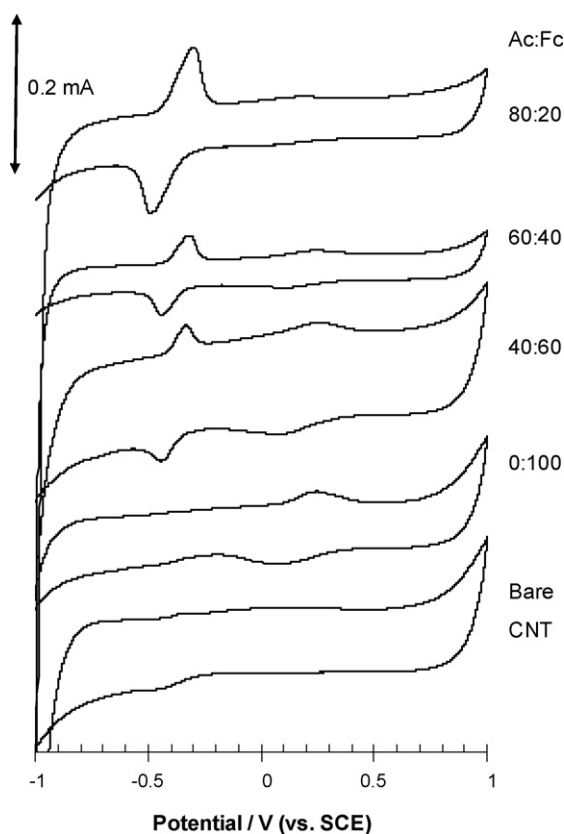


Fig. 4. The effect of varying the monomer feed ratios in the polymerisation process on the cyclic voltammetric response (scan rate 50 mV s^{−1}) of the copolymer-CNT layers when placed in a pH 4 buffer solution.

the CNT, the presence of iron can be clearly observed in the spectra, thus confirming that ferrocene is on the carbon nanotube surface.

Finally, we sought the effect on the electrochemical response of the polymer-CNT layer related to varying the ratio of vinylferrocene to vinylanthracene within the polymerisation process. Fig. 4 details the cyclic voltammetric response (scan rate = 50 mV s^{−1}) for various copolymer-CNT layers when placed in a pH 4 buffer solution prepared

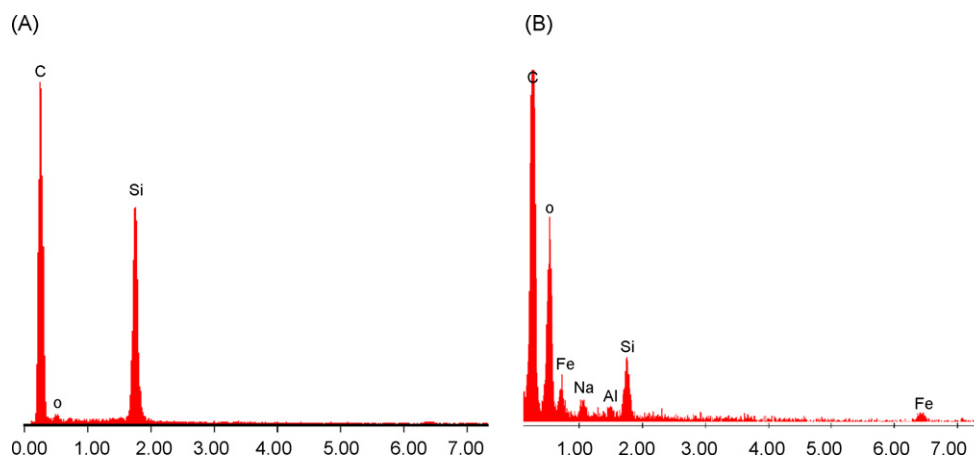


Fig. 3. EDAX spectra of (A) CNT and (B) p(Van_{40%}-co-VFc_{60%})-CNT layers.

with differing vinylanthracene:vinylferrocene monomer feed ratios. As expected, as the vinylanthracene concentration is lowered, the redox couple with peak currents at -0.40 V decreased with respect to the vinylferrocene redox couple wave at $+0.30$ V.

4. Conclusions

These results show the profound role that CNTs have on the anthracene moiety. The interaction between them has a significant effect on the linear response of anthracene redox potential with pH, which is hugely advantageous to its use as a pH sensor. This facile immobilisation procedure of copolymers containing π -electron-rich monomers with CNTs will allow analytical measurements in which the protonation state of the molecule can be tuned.

Congratulatory Statement

Dear Joe, Many happy returns on your 60th birthday, it was a privilege working with you in NMSU. Best wishes for the future.



Acknowledgement

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References

- [1] S. Iijima, *Nature* 354 (1991) 56.
- [2] R. Czerw, Z. Guo, P.M. Ajayan, Y.-P. Sun, D.L. Carroll, *Nano Lett.* 1 (2001) 423.
- [3] J. Zhang, J.K. Lee, Y. Wu, R.W. Murray, *Nano Lett.* 3 (2003) 403.
- [4] Y. Lin, B. Zhou, K.A. Fernando, P. Liu, L.F. Allard, Y.P. Sun, *Macromolecules* 36 (2003) 7199.
- [5] S.E. Kooi, U. Schlecht, M. Burghard, K. Kern, *Angew. Chem.* 114 (2002) 1409.
- [6] S.E. Kooi, U. Schlecht, M. Burghard, K. Kern, *Angew. Chem. Int.* 41 (2002) 1353.
- [7] M. Shim, N.W. Shi Kam, R.J. Chen, Y. Li, H. Dai, *Nano Lett.* 2 (2002) 285.
- [8] J. Chen, H. Liu, US Patent 6,905,667, B1 (2005).
- [9] B. McCarthy, J.N. Coleman, R. Czerw, A.B. Dalton, D.L. Carroll, W.J. Blau, *Synthetic Met.* 121 (2001) 1225.
- [10] J.H. Fan, D.B. Zhu, B.H. Chang, Z.W. Pan, S.S. Xie, *J. Appl. Polym. Sci.* 74 (1999) 2605.
- [11] M. Cochet, W.K. Maser, A.M. Benito, M.A. Callejas, M.T. Martinez, J.M. Benoit, *Chem. Commun.* 16 (2001) 1450.
- [12] M.J. O'Connell, P. Boul, L.M. Ericson, C. Huffman, Y. Wang, E. Haroz, *Chem. Phys. Lett.* 342 (2001) 265.
- [13] K.L. Robinson, N.S. Lawrence, *Anal. Chem.* 78 (2006) 2450.
- [14] K.L. Robinson, N.S. Lawrence, *Electroanalysis* 18 (2006) 677.
- [15] C. Amatore, M. Gareil, J.M. Saveant, *J. Electroanal. Chem.* 176 (1984) 377.
- [16] C. Amatore, M. Gareil, J.M. Saveant, *J. Electroanal. Chem.* 147 (1983) 1.
- [17] A.K. Covington, R.G. Bates, R.A. Durst, *Pure Appl. Chem.* 57 (1985) 531.
- [18] G.G. Wildgoose, H.C. Leventis, I. Streeter, N.S. Lawrence, S.J. Wilkins, L. Jiang, T.G.J. Jones, R.G. Compton, *Chem. Phys. Chem.* 5 (2004) 669.
- [19] K. Kelley, P.E. Pehrsson, L.M. Ericson, W. Zhao, *J. Nanosci. Nanotechnol.* 5 (2005) 1041.
- [20] M.S. Strano, *Nat. Mater.* 5 (2006) 433.
- [21] M. Kaempgen, S. Roth, *J. Electroanal. Chem.* 586 (2006) 72.
- [22] Q. Li, J. Zhang, H. Yan, M. He, Z.S. Liu, *Carbon* 42 (2004) 287.
- [23] R.J. Chen, Y. Zhang, D. Wang, H. Dai, *J. Am. Chem. Soc.* 123 (2001) 3838.
- [24] C.G.R. Heald, G.G. Wildgoose, L. Jiang, T.G.J. Jones, R.G. Compton, *Chem. Phys. Chem.* 5 (2004) 1794.
- [25] A.T. Masheter, P. Abiman, G.G. Wildgoose, E. Wong, L. Xiao, N.V. Rees, R. Taylor, G.A. Attard, R. Baron, A. Crossley, J.H. Jones, R.G. Compton, *J. Mater. Chem.* 17 (2007) 2616.
- [26] P. Abiman, A. Crossley, G.G. Wildgoose, J.H. Jones, R.G. Compton, *Langmuir* 23 (2007) 7847.

Review

Nanomaterial labels in electrochemical immunosensors
and immunoassaysGuodong Liu^{a,*}, Yuehe Lin^{b,**}^a Department of Chemistry and Molecular Biology, North Dakota State University, Fargo, ND 58105-5516, United States^b Pacific Northwest National Laboratory, Richland, WA 99352, United States

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In honor of the 60th Birthday of Professor Joseph Wang.

Abstract

This article reviews recent advances in nanomaterial labels in electrochemical immunosensors and immunoassays. Various nanomaterial labels are discussed, including colloidal gold/silver, semiconductor nanoparticles, and markers loaded nanocarriers (carbon nanotubes, apoferritin, silica nanoparticles, and liposome beads). The enormous signal enhancement associated with the use of nanomaterial labels and with the formation of nanomaterial–antibody–antigen assemblies provides the basis for ultrasensitive electrochemical detection of disease-related protein biomarkers, biothreat agents, or infectious agents. In general, all endeavors cited here are geared to achieve one or more of the following goals: signal amplification by several orders of magnitude, lower detection limits, and detecting multiple targets.

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Keywords: Nanomaterials; Nanoparticles; Immunosensors; Immunoassays; Carbon nanotubes; Biomarkers

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1. Introduction

Electrochemical immunosensors and immunoassays (EII) have recently attracted considerable interest because of their high sensitivity, low cost, and inherent miniaturization [1–6]. The principle of EII is based on a specific reaction of the

* Corresponding author. Tel.: +1 701 231 8697; fax: +1 701 231 8831.

** Corresponding author. Tel.: +1 509 376 0529; fax: +1 509 376 5106.

E-mail addresses: guodong.liu@ndsu.edu (G. Liu),
yuehe.lin@pnl.gov (Y. Lin).

antibody and antigen. For immunosensors, the immunologic materials are immobilized on an electrochemical transducer. For immunoassay, the immunological materials are immobilized on a solid supporting materials (microplate wells, magnetic beads, and polystyrene beads). After a sandwich or competitive immunoreaction, nanomaterial labels are attached to the transducer surface or supporting-material surface. Quantification is generally achieved by measuring the specific activity of a nanomaterial label after a releasing step or adding a substrate, i.e., its redox activities and enzyme activities. The novel characteristics of EII make them excellent candidates for clinical and point-of-care diagnosis of disease-related protein biomarkers. Disease biomarkers and biological agents are often present at ultra-low levels and require ultrasensitive methods for detection. Different strategies have been employed for amplifying the transducing signals of antibody–antigen interactions. Most conventional amplification strategies have relied on the use of labels, such as enzymes, electroactive molecules, redox complexes, and metal ions [1]. The emergence of nanotechnology is opening new horizons for the use of nanomaterial labels for signal amplification in EII [7–14]. The power and scope of such nanomaterials can be greatly enhanced by coupling them with immunoreactions and electrical processes (i.e., nanobioelectronics). The applications of nanomaterials in EII can be classified into two categories according to their functions: (1) nanomaterial-modified electrochemical transducers to facilitate antibody immobilization or improve electrochemical properties of transducers, such as low-background current, high signal to noise ratio, and fast electron transfer; (2) nanomaterial-bimolecular conjugates as labels for EII. In particular, nanomaterial labels are showing the greatest promise for developing ultrasensitive EII [7,14]. Antibodies (antigens) labeled with nanomaterials can retain their bioactivity and interact with their counterparts, and based on the electrochemical detection of those nanomaterials, the amount or concentration of analytes can be determined. The enormous signal enhancement associated with the use of nanomaterial amplifying labels and with the formation of nanomaterial–antibody–antigen assemblies provides the basis for ultrasensitive EII [7].

This article provides a review of the major advances and milestones in EII development based upon nanomaterial labels and their roles for biomarker detection. Particular attention will be given to new signal amplification and new emerging nanomaterial labels in EII. Such developments provide the pathway for diverse and exciting applications.

2. Nanomaterials used as EII labels

Many kinds of nanomaterials, including metal nanoparticles (gold, silver), semiconductor nanoparticles, enzyme-loaded carbon nanotubes (CNTs) and electroactive component-loaded nanovehicles (silica nanoparticle, polymer beads, and liposome beads), have been widely used in EII (Fig. 1). The common characteristics of these nanomaterial labels in EII are providing signal amplifications comparing to the traditional metal ion labels, enzyme labels and redox probe labels.

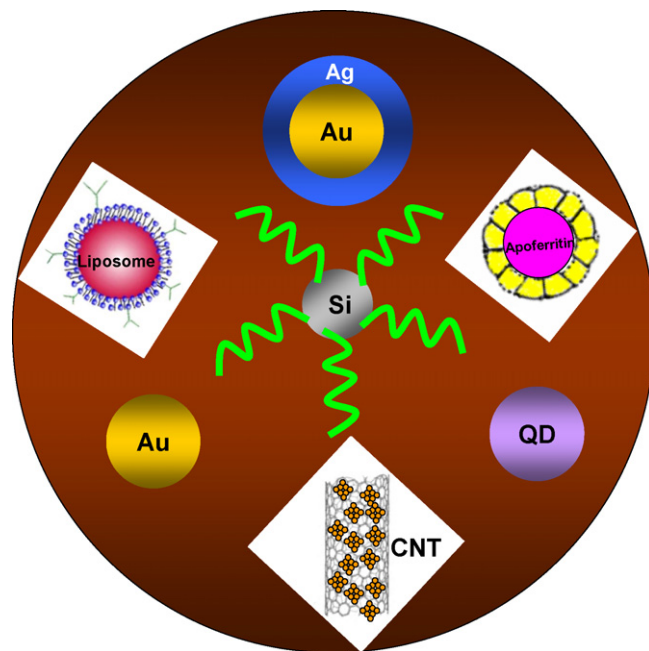


Fig. 1. Nanomaterial labels in electrochemical immunosensors and immunoassays.

2.1. Colloidal gold/silver

The applications of colloidal gold/silver in immunoassays date back to the early 1970s when 5–50 nm colloidal gold particles were first used as electron-dense probes in electron microscopy and thus enabled sensitive, high-resolution immunocytochemistry [15]. The subsequent development of silver-enhanced methods then allowed gold labels to provide very specific and sensitive immunocytochemistry. Because of the intrinsic electrochemical characteristics of gold and silver, colloidal gold/silver was introduced into the research fields of EII. The use of colloidal gold as an electrochemical label for voltammetric monitoring of protein interactions was pioneered in 2000 by Gonzalez-Garcia et al. [16] and Dequaire et al. [17]. Costa-Garcia's group thus demonstrated the monitoring of streptavidin–biotin interactions down to the 2.5 nM streptavidin level with adsorptive voltammetric measurements of the colloidal gold tags at a carbon-paste electrode [16]. Dequaire et al. reported a sensitive electrochemical immunoassay based on a colloidal gold label that was indirectly determined by anodic stripping voltammetry (ASV) at a single-use, carbon-based, screen-printed electrode (SPE) after oxidative gold metal dissolution in an acidic solution (Fig. 2) [17]. The method was evaluated for a noncompetitive heterogeneous immunoassay of an immunoglobulin G (IgG), and a concentration as low as 3×10^{-12} M was determined, which was competitive with a colorimetric enzyme-linked immunosorbent assay (ELISA) or with immunoassays based on fluorescent europium chelated labels. To avoid the acid-dissolution step, Liu et al. developed an electrochemical magnetic immunosensor based on magnetic beads and gold nanoparticle labels [18]. The captured gold nanoparticle labels on the immunosensor surface were directly quantified by electrochemical stripping analysis. The stripping signal of

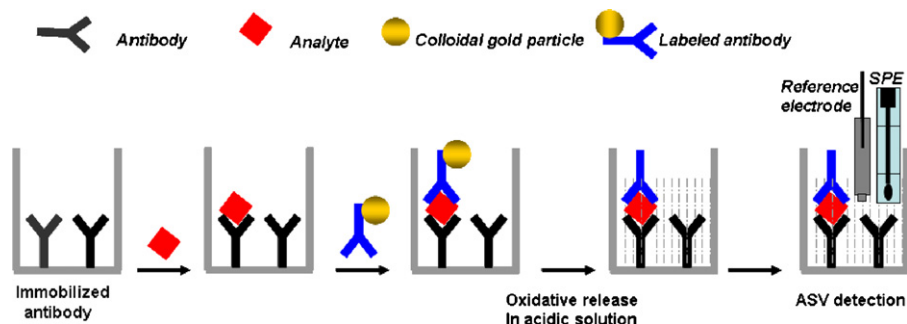


Fig. 2. Schematic representation of the noncompetitive heterogeneous electrochemical immunoassay based on a colloidal gold label (based on Ref. [17] with permission).

gold nanoparticles is related to the concentration of target IgG in the sample solution. The detection limit of $0.02 \mu\text{g mL}^{-1}$ of IgG was obtained under optimum experimental conditions. Ambrosi et al. reported double-codified gold nanolabels for simultaneous electrochemical and optical immunoassays [19]. A built-in magnet graphite-epoxy-composite electrode allowed a sensibly enhanced adsorption and electrochemical quantification of the specifically captured gold nanoparticle labels on the paramagnetic bead surface. The detection limits for this double-codified nanoparticle-based assay were 52 and 260 pg of human IgG/mL for the spectrophotometric (horseradish peroxidase [HRP]-based) and electrochemical (AuNP-based) detections, respectively, much lower than those typically achieved by ELISA tests.

To further enhance the sensitivity of gold nanoparticle-label-based EII, various methods and technologies have been developed. For example, the nanoparticle-promoted precipitation of silver on gold nanoparticle labels was used to amplify the transduction of antibody–antigen biorecognition events [20–23]. Chu et al. reported an electrochemical immunoassay based on silver on colloidal gold labels, which, after silver metal dissolution in an acidic solution, was indirectly determined by ASV at a glassy-carbon electrode [20]. The method was evaluated for a noncompetitive heterogeneous immunoassay of using IgG as a model analyte. The anodic stripping peak current of silver depended linearly on the IgG concentration over the range of $1.66\text{--}27.25 \text{ ng mL}^{-1}$ in a logarithmic plot. A detection limit as low as 1 ng mL^{-1} (i.e., $6 \times 10^{-12} \text{ M}$) human IgG was achieved. The method was applied to detect schistosoma japonicum antibodies (SjAb) in rabbit serum [21]. A detection limit as low as 3.0 ng/mL SjAb was achieved. A potentiometric immunosensor based on gold nanoparticle labels and an ion-selective microelectrode was reported by Chumbimuni-Torres et al. [22]. This immunosensor is based on a sandwich immunoassay where the target mouse IgG antigen is captured by the primary anti-mouse gold substrate modified by IgG antibodies, followed by adding a secondary anti-mouse IgG antibody conjugated to gold nanoparticle tags and by catalytic silver enlargement onto the gold labels. The precipitated silver is oxidatively dissolved with hydrogen peroxide to yield dilute electrolyte backgrounds for the potentiometric detection of the released silver ions with a polymer-membrane, silver, ion-selective microelectrode. Silver-enhanced gold nanoparticle

labels were also used to develop conductive immunosensors. For example, Velez and Kaler reported a conductive immunosensor using antibody-functionalized latex spheres and a microelectrode gap [23]. A sandwich immunoassay led to the binding of a secondary gold-nanoparticle-labeled antibody on the latex spheres that were located in the gap, followed by catalytic deposition of a silver layer “bridging” the two electrodes. Such a formation of conductive paths across interdigitated electrodes led to a measurable conductive signal and enabled the ultrasensitive detection of human IgG down to the 0.2 pM level. The method holds promise for creating miniaturized on-chip protein arrays.

A novel dendritic amplification procedure has been developed for quartz crystal microbalance (QCM) immunosensing by applying antibody-functionalized Au nanoparticles as the primary amplifying probe and a dendritic-type immunocomplex of protein A- and antibody-modified Au nanoparticles as the secondary amplifying probe [24]. The primary amplification of the recognition process is implemented via the interaction of the sensing interface with the antibody-functionalized Au nanoparticles, and the secondary dendritic amplification is performed through interaction with the immunocomplex of protein A- and antibody-modified Au nanoparticles. The frequency decreases of the primary-amplified and the secondary-amplified sensing process are observed to be linearly dependent upon the IgG concentration in the range of 10.9 ng mL^{-1} to $10.9 \mu\text{g mL}^{-1}$ with a detection limit of 3.5 ng mL^{-1} , while in the absence of the amplification processes, the antigen-antibody recognition event can only be detected for an IgG concentration as high as $10.9 \mu\text{g mL}^{-1}$.

Recently, Mao et al. presented a new method based on cyclic accumulation of gold nanoparticles for detecting human immunoglobulin G (IgG) by ASV [25]. The dissociation reaction between dethiobiotin and avidin in the presence of biotin provides an efficient means for the cyclic accumulation of gold nanoparticles used for the final analytical quantification. The anodic peak current increases gradually with the increasing accumulation cycles. Five cycles of accumulation are sufficient for the assay. The low background of the proposed method is a distinct advantage, providing a possibility for determining at least 0.1 ng/mL human IgG.

An electrochemical immunoassay method based on Au nanoparticle-labeled immunocomplex enlargement was

reported by Zhou et al. [26]. When the aggregates formed from nano-Au labeled goat-anti-human C-3 and nano-Au labeled rabbit-anti-goat IgG were immobilized on the electrode surface by the sandwich method (antibody/antigen/aggregate), the electrochemical signal of the electrode was enlarged greatly. The reported immunosensor could quantitatively determine complement C-3 in the range of 0.12, similar to 117.3 ng mL⁻¹, and the detection limit was 0.02 ng mL⁻¹.

Liao et al. reported an amplified electrochemical immunoassay by autocatalytic deposition of Au³⁺ onto gold nanoparticle labels [27]. By coupling the autocatalytic deposition with square-wave stripping voltammetry, enlarged gold nanoparticles were used as labels on goat anti-rabbit immunoglobulin G (GaRIgG-Au) and, thus, the rabbit immunoglobulin G (RIgG) analyte could be determined quantitatively. The detection limit was 0.25 pg mL⁻¹ (1.6 fM), which is three orders of magnitude lower than that obtained by a conventional immunoassay using the same gold nanoparticle labels.

Recently Das et al. reported an ultrasensitive electrochemical immunosensor using the gold nanoparticle labels as electrocatalysts [28]. In this case, the gold nanoparticle labels were attached to the immunosensor surface (indium tin oxide as substrate electrode) by sandwich immunoreaction; the signal amplification was achieved by catalytic reduction of *p*-nitrophenol (NP) to paminophenol (AP) and chemical reduction of *p*-quinone imine (QI) to AP by NaBH₄ (Fig. 3). Such dual amplification events gave a 1 fg mL⁻¹ detection limit, and its linear range of measurement ranged from 1 fg mL⁻¹ to 10 μg mL⁻¹, which covered a 10-order concentration range.

2.2. Semiconductor nanoparticles

Owing to their unique (size-tunable fluorescent) properties, semiconductor (quantum dot, QD) nanoparticles have generated considerable interest for optical biodetection [29]. The intrinsic redox properties and the sensitive electrochemical stripping analysis of the metal components of semiconductor nanoparticles (CdS, PbS, and ZnS) cause the labels in the electrochemical biosensors to be very sensitive [7,14]. The concept was first demonstrated by Wang's group using semi-

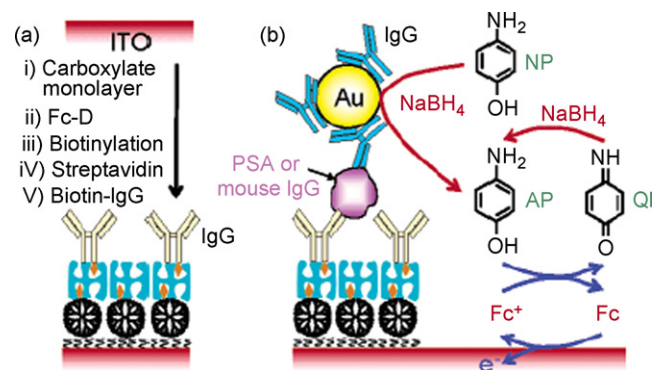


Fig. 3. (a) Schematic representation of the preparation of an immunosensing layer. (b) Schematic view of electrochemical detection of mouse IgG or PSA (reproduced from Ref. [28] with permission).

conductor nanoparticle labels for the electrochemical DNA hybridization assay [30,31], and then it was extended in EII [32]. Liu et al. [32] reported an electrochemical immunoassay protocol for the simultaneous measurements of multiple protein targets based on the use of different semiconductor nanoparticle tracers (CdS, ZnS and PbS). Carbamate linkage is used for conjugating the hydroxyl-terminated semiconductor nanoparticles with the secondary antibodies. The multianalyte electrical sandwich immunoassay involves a dual binding event, based on antibodies linked to the nanocrystal tags and magnetic beads (Fig. 4). Each biorecognition event yields a distinct voltammetric peak whose position and size reflects the identity and level, respectively, of the corresponding antigen. The multi-protein electrical detection capability is coupled to the amplification feature of electrochemical stripping transduction (to yield fmol detection limits) and with an efficient magnetic separation (to minimize nonspecific adsorption effects). The concept has been demonstrated for a simultaneous immunoassay of α2-microglobulin, IgG, bovine serum albumin, and C-reactive protein in connection with ZnS, CdS, PbS, and CuS colloidal crystals, respectively. Hong et al. reported a similar approach using commercial QD (ZnS@CdS) labels for the electrochemical immunoassay of a protein biomarker, interleukin-1α (IL-1α) [33]. After sandwich immunoreaction, QD labels were attached

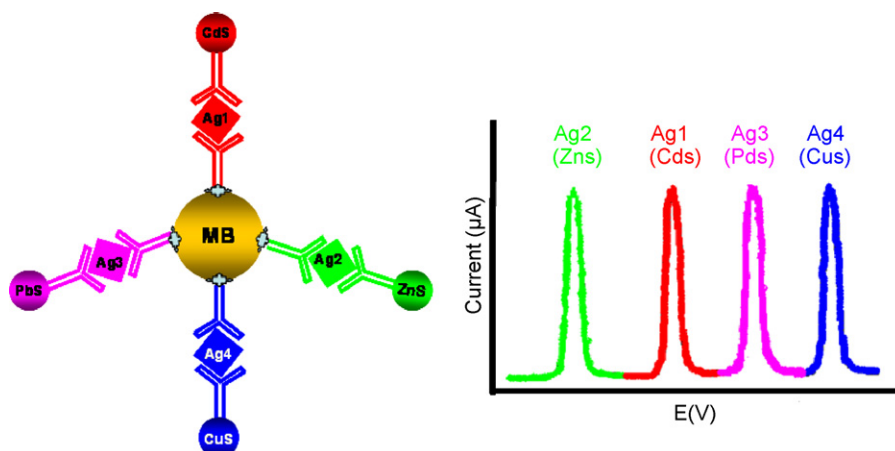


Fig. 4. Multiprotein electrical detection protocol based on different metallic sulfide nanoparticle labels (based on Ref. [32] with permission).

to the IL-1 α antibody-coated magnetic beads by the formed antibody–antigen immunocomplex. The concentration of IL-1 α was determined by electrochemical stripping analysis of the cadmium component of the captured QD labels after an acid-dissolution step. The voltammetric response is highly linear over the range of 0.5–50 ng mL⁻¹ IL-1 α , and the limit of detection is estimated to be 0.3 ng mL⁻¹ (18 pM).

Recently, Hansen et al. reported using a QD/aptamer-based ultrasensitive electrochemical biosensor to detect multiple protein targets [34]. The protocol is based on a simple single-step displacement assay involving the co-immobilization of several thiolated aptamers, along with binding of the corresponding QD-tagged proteins on a gold surface, adding the protein sample, and monitoring the displacement through electrochemical detection of the remaining nanocrystals. Such electronic transduction of aptamer–protein interactions is extremely attractive for meeting the low power, size, and cost requirements of decentralized diagnostic systems. Unlike two-step sandwich assays used in the above QD-based electrochemical immunoassays, the new aptamer biosensor protocol relies on a single-step displacement protocol. This biosensor allows assays of samples with target concentrations that are 3–4 orders of magnitude with a detection limit of 20 ng L⁻¹ (0.5 pM). Such a detection limit corresponds to 54.5 attomole (2 pg) in the 100 μ L sample.

QD has also been used as a label for potentiometric immunoassays [35]. Thurer reported the potentiometric bioanalysis of proteins in a microtiter plate format with semiconductor nanocrystal labels. After sandwich immunoreaction on the microtiter plate, the captured CdSe QDs were found to be easily dissolved/oxidized in a matter of minutes with hydrogen peroxide. The released Cd²⁺ ions were measured by Cd²⁺ selective micropipette electrodes. The potentiometric protein immunoassay exhibits a log-linear response ranging from 0.15 to 4.0 pmol of IgG, with a detection limit of <10 fmol in 150 μ L sample wells.

2.3. Carbon nanotubes

CNTs have been explored extensively in electroanalytical chemistry research fields [see reviews, 36–40]. Because of their big surface area-to-weight ratio, excellent mechanical properties, and fast electron-transfer capabilities, CNTs have been widely used to prepare electrochemical sensors and biosensors and study the electron-transfer characteristics of proteins.

Recently, Wang et al. investigated their novel applications by using CNTs as labels for ultrasensitive electrochemical immunoassays [41]. In this case, CNTs were used as a “carrier” to load numerous enzyme tracers by covalent functionalization of their surface (Fig. 5). A coverage of around 9600 enzyme molecules per CNT (1 μ m length) was estimated. The numerous enzyme-loaded CNT super labels were applied for the magnetic beads-based electrochemical immunoassay of IgG, and a detection limit of 500 fg mL⁻¹ (160 zmol in 25 μ L samples) was obtained. A 100-fold enhancement in the resulting electrochemical signal was observed from the enzyme-CNT label when compared to a system consisting of a single-enzyme label. Further sensitivity enhancement was achieved with a layer-by-layer (LBL) assembly of multilayer enzyme films on the CNT template [42]. Such coupling of CNT labels and protein multiple architectures was shown to maximize the ratio of enzyme tracers per binding event, to offer a remarkably high amplification factor, and hence to achieve an extremely low detection limit down to 2000 protein molecules (67 aM). Rustling’s team reported on CNT “forest” amperometric immunosensor platforms with multi-labeled secondary antibody–CNT bioconjugates for highly sensitive detection of a cancer biomarker in serum and tissue lysates [43]. Greatly amplified sensitivity was attained by using bioconjugates featuring HRP labels and secondary antibodies (Ab₂) linked to CNTs at a high HRP/Ab₂ ratio. This approach provided a detection limit of 4 pg mL⁻¹ (100 amol mL⁻¹), for prostate-specific antigen (PSA) in 10 μ L of undiluted calf serum, a mass detection limit of 40 fg.

2.4. Apoferritin nanovehicles

Recently, we used apoferritin as a template to prepare nanoparticle labels for a highly sensitive electrochemical immunoassay of protein [44–47]. Apoferritin consists of a spherical protein shell (around 12.5 nm in diameter) composed of 24 subunits surrounding an aqueous cavity with a diameter of about 8 nm that is capable of accommodating around 4500 iron atoms [48]. The protein cage of apoferritin can be disassociated into 24 subunits at low pH (2.0) and reconstituted at a high pH (8.5) environment. There are 14 channels connecting the outside of apoferritin with its interior, among which six are hydrophobic channels, and eight are hydrophilic channels. Based on its unique properties, two types of apoferritin nanoparticle labels, including the redox probe-loaded apofer-

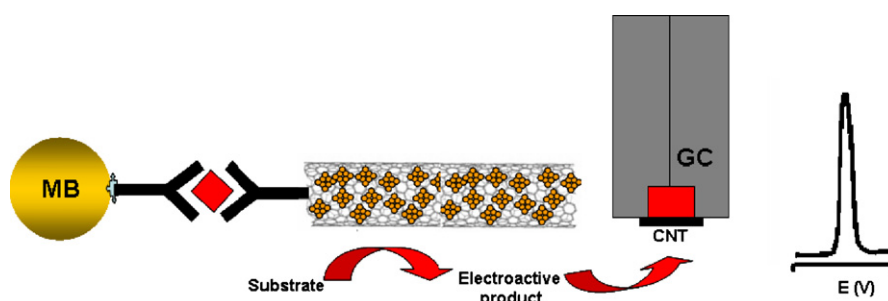


Fig. 5. Ultrasensitive electrochemical immunoassay of protein based on the numerous enzymes loaded with CNT labels (based on Ref. [41] with permission).

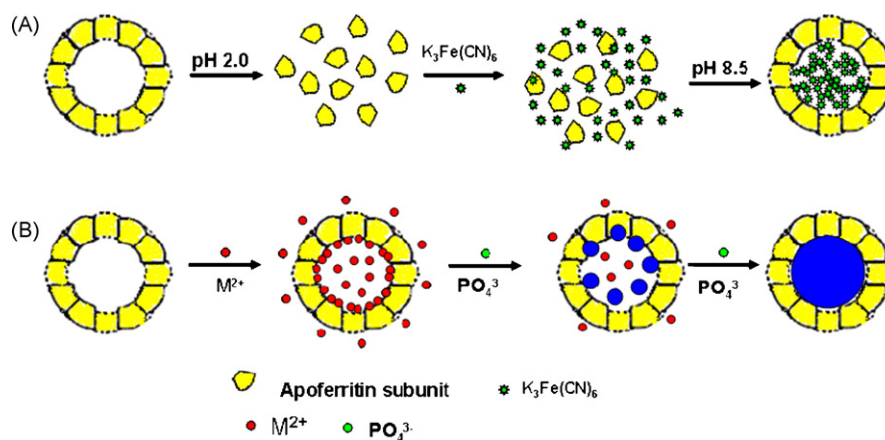


Fig. 6. (A) Preparation of $K_3Fe(CN)_6$ -loaded apoferritin nanoparticle labels (based on Ref. [45] with permission) and (B) apoferritin template synthesis metallic phosphate nanoparticle tag (based on Refs. [46,47] with permission).

ritin and metallic phosphate-loaded apoferritin, were developed (Fig. 6). The concept was first demonstrated by loading hexacyanoferrate in the cavity of apoferritin for the electrochemical immunoassay of an IgG model analyte [45]. The preparation of hexacyanoferrate-loaded apoferritin is illustrated in Fig. 6A. Briefly, apoferritin was dissociated into subunits at pH 2 and then reconstituted at pH 8.5, thereby trapping hexacyanoferrate in solution within its interior. Around 150 hexacyanoferrates were loaded into an apoferritin. The hexacyanoferrate-loaded apoferritin was thus modified with biotin and used as a label for a magnetic bead-based sandwich immunoassay of IgG. The electrochemical response of the target was obtained by square-wave voltammetric measuring of the released hexacyanoferrate tracers from the captured nanoparticle labels. A detection limit of 0.08 ng mL^{-1} (0.52 pM) was obtained in connection with the 60 min immunoreaction time. This detection limit corresponds to 26 attomole in the $50 \mu\text{L}$ sample solution.

Inspired by the use of semiconductor nanoparticle labels in the electrochemical bioassay, we developed a simple and facile templated synthesis strategy based on an apoferritin template to prepare uniform-size, metal phosphate nanoparticle labels for a highly sensitive electrochemical immunoassay (Fig. 6B) [46]. Comparing the semiconductor nanoparticle tags, new metallic phosphate nanoparticle tag is easy to prepare (simply by diffusion or adjusting the pH of the solution) and functionalize (protein shell). Releasing metal components from the nanoparticle was performed in the mild condition, pH 4.6 acetate buffer, instead of a strong acid solution (1 M HNO_3 to dissolve the semiconductor nanoparticle tags). New nanoparticle was used as a label for the electrochemical immunoassay of the tumor necrosis factor α (TNF- α) protein biomarker with a detection limit of 2 pg mL^{-1} (77 fM), that is, 3.9 attomols or 2.33×10^6 TNF- α biomarker molecules in a $50 \mu\text{L}$ sample, which is lower than that of the semiconductor nanoparticle tag. The ability to simultaneously measure multiple proteins in a single assay was also demonstrated by detecting TNF- α and macrophage chemotactic protein-1 protein biomarkers with cadmium phosphate and lead phosphate nanoparticle tags.

Except for the preparation of single-metal-component, metal phosphate nanoparticle tags, compositionally (multiple metals)

encoded nanoparticle tags were also prepared by the apoferritin template [47]. By incorporating different predetermined levels of multiple metal ions, such compositionally encoded nanoparticles can lead to a large number of recognizable voltammetric signatures and hence to a reliable detection of a larger number of protein biomarkers. Each nanoparticle thus yields a characteristic multi-peak voltammogram, whose peak potentials and current intensities reflect the identity of the corresponding biomolecule target. The apoferritin template synthesis of the encoded nanoparticle provides a simpler, faster approach to prepare electrochemical nanoparticle labels for diverse applications.

2.5. Liposome

Liposomes are composed of a lipid bilayer with the hydrophobic chains of the lipids forming the bilayer and the polar headgroups of the lipids oriented towards the extravesicular solution and inner cavity [49]. The sizes of the liposomes vary, ranging from nanometers to several micrometers, which depends on the synthesis conditions [49]. Owing to its high surface area, large internal volume, and capability to conjugate bilayer lipids with a variety of biorecognition elements, liposomes have been widely used as bioassay labels by encapsulating enzymes, fluorescent dyes, electrochemical and chemiluminescent markers, DNA, RNA, ions, and radioactive isotopes [50,51]. While excellent reviews of the uses of liposomes in immunoassays are available in the literature [49,50], we focus on the applications of liposomes as labels in EII. Fig. 7 displays the typical liposome label-based electrochemical immunoassay. Most of the early works are based on ion-encapsulated liposome labels and ion-selective electrodes for serum protein analysis [52–54]. For example, D’Orazio and Rechnitz used trimethylphenylammonium ion (TMPA^+)-loaded sheep red-blood-cell ghosts (which act in the same capacity as liposome) to quantitate the complement enzymes present in serum samples by using a TMPA^+ ion selective electrode [52]. They were later able to adapt this technique to indirectly detect antibodies in bovine serum albumin. Similarly, Shiba et al. demonstrated the potentiometric determination of tetrapentylammonium ion (TPA^+) with a TPA^+

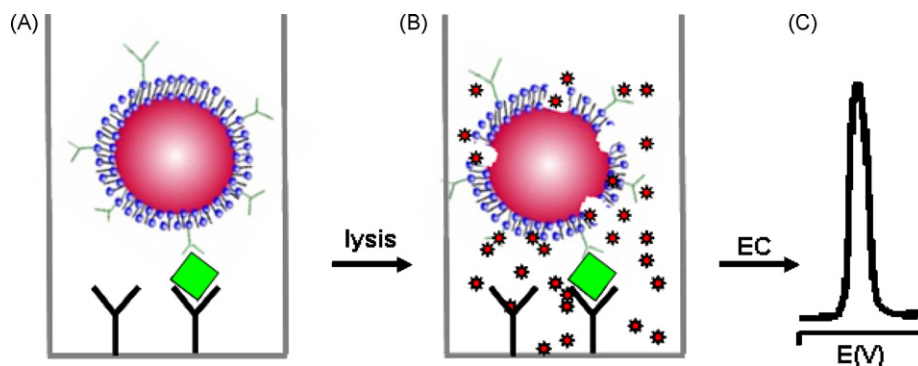


Fig. 7. Schematic of an electrochemical immunoassay based on markers loaded with liposome labels: (A) immunocapturing liposome labels; (B) lysis to release the markers; (C) electrochemical detection.

ion-selective electrode upon complement disruption of TPA^+ of the TPA^+ -loaded liposomes [53]. Shiba et al. first used potassium ion-loaded liposomes and a potassium ion-selective electrode for monitoring the complement-mediated immune lysis reaction [54].

Liposomes encapsulating electroactive molecules, such as ferrocyanide and ascorbic acid, have been used as labels for EII [55,56]. Kannuck reported ferrocyanide-encapsulated liposome for selective and sensitive (0.1 nM) determination of immuno-agents in serum matrices [55]. Ferrocyanide was encapsulated in the cavity of liposomes at concentrations of approximately 10^4 molecules/liposome. The ferrocyanides were released from within the liposome by either the addition of surfactant or the complement lysis of the membrane and were monitored by differential pulse voltammetry on a polymer-modified electrode. Liposomes encapsulating ascorbic acid have been used in a competitive assay format for the pesticide atrazine using both lateral and horizontal flow formats and amperometric detection following Triton X-100-induced lysis [56]. The signal response was 1–3 min, and the sensitivity of the measurements in tap water was below $1 \mu\text{g L}^{-1}$ of atrazine and terbutylazine. Lee et al. reported a disposable liposome immunosensor for theophylline, combining an immunochromatographic membrane and a thick-film electrode [57]. An anti-theophylline antibody was immobilized in an antibody competition zone, and hexacyanoferrate(II)-loaded liposomes were immobilized in a signal generation zone. When a theophylline sample solution was applied to the immunosensor pre-loaded with theophylline–melittin conjugate in a sample loading zone, the theophylline and theophylline–melittin conjugate migrated through the anti-theophylline antibody zone where competitive binding occurs. Unbound theophylline–melittin conjugate further migrated into the signal generation zone where it disrupted the liposomes to release the electroactive hexacyanoferrate(II), which was then detected amperometrically. The detection limit of $5 \mu\text{g mL}^{-1}$ enabled the immunosensor to be used to monitor theophylline over the clinically relevant ranges ($10\text{--}20 \mu\text{g mL}^{-1}$) with a one-step assay within 20 min.

Liposome encapsulating enzymes, such as HRP, have been used as labels to develop sensitive electrochemical immunosensors [58]. For example, Haga reported a liposome immunosensor

for the detection of theophylline. The immunosensor is composed of a Clark-type oxygen electrode and HRP encapsulated liposomes. Sample theophylline and theophylline-tagged liposomes competed for antibody sites. Binding to the antibody initiated the activation of the complement, which lysed the liposomes and resulted in the release of the entrapped horseradish peroxidase. The released HRP catalyzed the conversion of NADH to NAD^+ , and the corresponding depletion of oxygen was monitored by an oxygen electrode. The authors reported a detection limit for theophylline of 0.72 ng/mL .

Recently, Chen et al. developed a novel piezoelectric immunoagglutination assay (PEIA) technique with antibody-conjugated liposome for direct quantitative detection of human immunoglobulin G (hIgG) [59]. This technique is based on specific agglutination of antibody-coated liposome particles in the presence of the corresponding antigen, which can be monitored by the frequency shift of a piezoelectric device. The frequency responses of the liposome-based PEIA are linearly correlated to hIgG concentration in the range of $0.05\text{--}6 \mu\text{g mL}^{-1}$ with a detection limit of 50 ng mL^{-1} .

2.6. Silica nanoparticles

Silica nanoparticles have been successfully used in bioimaging, optical bioassays, drug delivery, diagnosis, and therapeutic applications [60–64]. Recently, we developed an “electroactive” silica nanoparticle in which poly(guanine) (poly[G]) was used to functionalize silica nanoparticles, and then we demonstrated that the poly[G]-functionalized silica NPs could serve as a biological label for a sensitive electrochemical immunoassay [65,66]. The “electroactive” silica nanoparticle label was prepared by covalently binding poly[G] and avidin to the silica NP surface by using the conventional coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide. It was found that there are ~ 60 strands of poly[G]₂₀ per silica nanoparticle. Accordingly, the average surface coverage of poly[G]₂₀ on a silica surface was determined to be $\sim 8.5 \times 10^{12} \text{ molecules cm}^{-2}$. The functionalized silica NPs could be used as a label for an amplified electrochemical immunoassay. The principle of an electrochemical immunoassay based on a poly[G]-functionalized silica NP label is shown

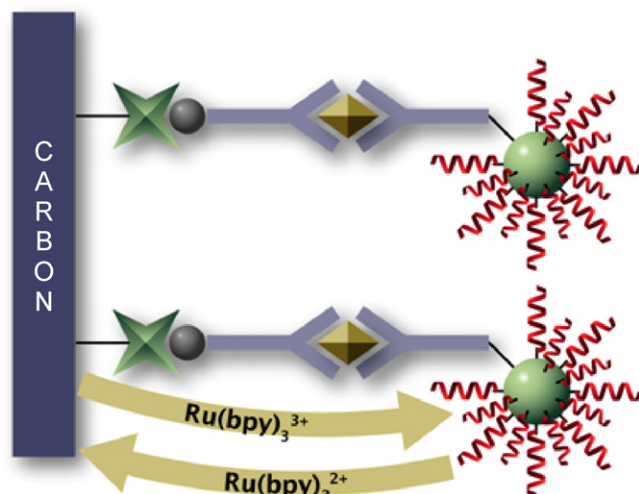


Fig. 8. Electrochemical immunosensor based on the poly[guanine]-coated silica nanoparticle (reproduced from Ref. [65] with permission).

in Fig. 8. After a complete sandwich immunoassay, a solution of $\text{Ru}(\text{bpy})_3^{2+}$ is added, and the catalytic current resulting from guanine oxidation is measured. This current is proportional to the amount of guanine in the vicinity of the electrode, which in turn depends on the concentration of target analyte in the original sample.

The application was first demonstrated by using IgG as model protein [65]. It was found that the current was proportional to the logarithm of IgG concentration. The limit of detection for this immunosensor (based on a signal-to-noise ratio $S/N=3$) is estimated to be about 0.2 ng mL^{-1} (about 1.3 pM), which corresponds to about 39 aM mouse IgG in a $30 \text{ }\mu\text{L}$ sample solution. The developed electroactive silica nanoparticle label was also used to detect protein biomarker, $\text{TNF-}\alpha$ [66]. After optimizing the experimental parameters (e.g., concentration of $\text{Ru}(\text{bpy})_3^{2+}$, incubation time of $\text{TNF-}\alpha$, etc.), the detection limit for $\text{TNF-}\alpha$ was found to be $5.0 \times 10^{-11} \text{ g mL}^{-1}$ (2.0 pM), which corresponds to 60 attomol of $\text{TNF-}\alpha$ in $30 \text{ }\mu\text{L}$ of sample.

2.7. Other labels

Except for the nanomaterial labels described above, micrometer size materials, such as polystyrene beads and microcrystals, have been used as labels for highly sensitive electrochemical immunoassays. For example, Wang et al. reported on nucleic acid-coated polystyrene bead labels for an amplified electrochemical sandwich immunoassay of proteins [67]. This involves a sandwich immunoassay based on two antibodies linked to magnetic beads and DNA-functionalized polystyrene (PS) spheres, followed by the alkaline release of the oligonucleotide strands from the beads, the acidic dipurination of the released DNA, and adsorptive chronopotentiometric stripping measurements of the free nucleobases at a pyrolytic graphite electrode transducer (Fig. 9). The coupling of carrier-sphere amplifiers (7.5×10^4 oligonucleotides [containing 25 guanines] per sphere) with the preconcentration feature of electrochemical stripping detection leads to an extremely low detection limit of 2 pg mL^{-1} (13 fM). A similar use of different oligonucleotide tracers – based on different guanine-to-adenine ratios – should lead to an attractive multiplex protein assay. A ferrocene microcrystal was also used as a biolabel for the amplified electrochemical immunoassay [68]. In this case, LBL technology was employed to encapsulate electrochemical signal-generating microcrystals (ferrocene microcrystals). The encapsulated microcrystals were conjugated with antibody molecules through the adsorption process for sandwich immunoassays. The capture of the antibody-conjugated ferrocene microcrystal labels was followed by the release of a large amount of the redox marker through the capsule wall (by a releasing agent) and led to highly sensitive amperometric biodetection with a detection limit of $2.82 \text{ }\mu\text{g L}^{-1}$.

Recent efforts focused on developing nanoparticle encoded spheres, submicrometer metallic microrods, and metal or alloy nanowires [29,70–73]. Such materials have been used as tags for product identification [69], an electrochemical DNA hybridization assay [71], and an optical bioassay [29,72,73]. For example, indium/gold nanowire tags prepared by electrodeposition indium and gold into the pores of an alumina membrane

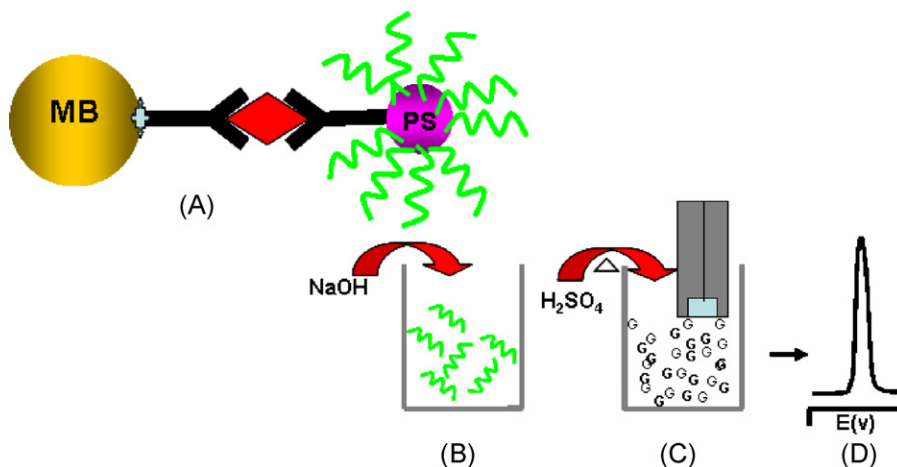


Fig. 9. Use of oligonucleotide-loaded polystyrene sphere labels for the amplified electrochemical immunoassay of proteins: (A) immunocapturing oligonucleotide-loaded polystyrene spheres to a magnetic bead; (B) release of the oligonucleotides from the sphere using 0.05 M NaOH ; (C) acid dipurination; (D) detection of the released purine-based at a graphite electrode (based on Ref. [67] with permission).

offered a significantly low detection limit (250 zmol) for the electrochemical DNA hybridization assay [69]. Semiconductor nanoparticle-encoded polystyrene beads [29] and metallic striped nanowires have been used as tags for the multiplex optical immunoassay of proteins, DNA, and pathogens [72,73]. Because of its big size (micrometer length), the content of metal in metallic nanowire is much more than that of the reported semiconductor nanoparticles. It will be possible to use such metallic nanowire tags for ultrasensitive electrochemical immunoassays.

3. Conclusion and outlook

This review has summarized the recent advances of nanomaterial labels in EII. The unique and attractive properties of nanomaterials have paved the way for the development of highly sensitive electrochemical diagnosis devices. The studies described above demonstrate the broad potential of bioconjugated nanomaterials for the amplified electrochemical transduction of antibody–antigen recognition events. The remarkable sensitivity of the new nanomaterial-label-based sensing protocols opens the possibility for detecting disease markers, biothreat agents, or infectious agents that cannot be measured by conventional methods. Such highly sensitive biodection schemes could provide for early detection of diseases or a warning of a terrorist attack. The use of super tags, such as electroactive-marker-loaded polymer beads and submicrometer metallic strips will further amplify the electrochemical signal of EII and thus bring about a lower detection limit. However, the challenges for current research are (1) “How will the nanomaterial-label-based EII technologies work in clinical samples, such as blood, plasma?” and (2) “Will it be possible to apply this technology to point-of-care and clinical diagnosis?” Future innovative research is expected to lead to advanced nanomaterial-based EII, which, coupled with other major technological advances, such as lateral-flow, test-strip technology and electronics technology, will result in powerful, easy-to-use, hand-held devices for fast, sensitive, and low-cost biomarker detection.

Congratulations

From G. Liu

I would like to congratulate Prof. Wang on his 60th birthday. HAPPY BIRTHDAY, Dr. Wang! Millions of thanks for your guidance, encouragement, and support during my career development! I am looking forward to reading your 1000th wonderful paper! Guodong.



From Y. Lin

I first met Dr. Wang when I joined his Biosensor group at Las Cruces in 1991 as a postdoctoral researcher working on organic-phase enzyme biosensors. The time I worked with Joe during 1991–1992 is unforgettable to me and is very important to my career development. Since I joined Pacific Northwest National Laboratory in 1997, Joe has been a valuable long-term collaborator of mine in research of lab-on-a-chip and carbon nanotubes biosensors. Whether working with students or colleagues, Joe is an excellent role model: he is a creative, diligent, and productive scientist. Many thanks to you, Joe, for your inspiration and encouragement over the years, and HAPPY 60th Birthday! Yuehe.



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References

- [1] A. Warsinke, A. Benkert, F.W. Scheller, *Fresenius J. Anal. Chem.* 366 (2000) 622.
- [2] A.L. Ghindilis, P. Atanasov, M. Wilkinst, E. Wilkins, *Biosens. Bioelectron.* 13 (1998) 113.
- [3] J. Lin, H. Ju, *Biosens. Bioelectron.* 20 (2005) 1461.
- [4] N.R. Stradiotto, H. Yamanaka, M.V.B. Zandoni, *J. Braz. Chem. Soc.* 14 (2003) 159.
- [5] C.A. Marquette, L.J. Blum, *Biosens. Bioelectron.* 21 (2006) 1424.
- [6] E. Bakker, Y. Qin, *Anal. Chem.* 78 (2006) 3965.
- [7] J. Wang, *Electroanalysis* 19 (2007) 769.
- [8] J. Wang, *Small* 1 (2005) 1036.
- [9] J. Wang, *Biosens. Bioelectron.* 21 (2006) 1887.
- [10] E. Katz, I. Willner, J. Wang, *Electroanalysis* 16 (2004) 19.
- [11] D. Hernandez-Santos, M.B. Gonzalez-Garcia, A.C. Garcia, *Electroanalysis* 14 (2002) 1225.

- [12] X. Luo, A. Morrin, A.J. Killard, M.R. Smyth, *Electroanalysis* 18 (2006) 319.
- [13] E. Katz, I. Willner, *Angew. Chem. Int. Ed.* 43 (2004) 6042.
- [14] G. Liu, J. Wang, H. Wu, Y. Lin, Y. Lin, *Electroanalysis* 19 (2007) 777.
- [15] M.A. Hayatt (Ed.), *Colloidal Gold-Principles, Methods and Applications*, Academic Press, San Diego, 1989.
- [16] M.B. Gonzalez-Garcia, C. Fernandez-Sanchez, A. Costa-Garcia, *Biosens. Bioelectron.* 15 (2000) 315.
- [17] M. Dequaire, C. Degrand, B. Limoges, *Anal. Chem.* 72 (2000) 5521.
- [18] G. Liu, Y. Lin, J. Nanosci. Nanotechnol. 5 (2005) 1060.
- [19] A. Ambrosi, M.T. Castaneda, A.J. Killard, M.R. Smyth, S. Alegret, A. Merkoci, *Anal. Chem.* 79 (2007) 5232.
- [20] X. Chu, X. Fu, K. Chen, G. Shen, R. Yu, *Biosens. Bioelectron.* 20 (2005) 1805.
- [21] X. Chu, Z. Xiang, X. Fu, S. Wang, G. Shen, R. Yu, *J. Immunol. Methods* 301 (2005) 77.
- [22] K.Y. Chumbimuni-Torres, Z. Dai, N. Rubinova, Y. Xiang, E. Pretsch, J. Wang, E. Bakker, *J. Am. Chem. Soc.* 128 (2006) 13676.
- [23] O.D. Velev, E.W. Kaler, *Langmuir* 15 (1999) 3693.
- [24] X. Chu, Z. Zhao, G. Shen, R. Yu, *Sens. Actuator B* 114 (2006) 696.
- [25] X. Mao, J.H. Jiang, J.W. Chen, Y. Huang, G.L. Shen, R.Q. Yu, *Anal. Chim. Acta* 557 (2006) 159.
- [26] G. Zhou, J. Li, J. Jiang, G. Shen, R. Yu, *Acta Chim. Sin.* 63 (2005) 2093.
- [27] K. Liao, H. Huang, *Anal. Chim. Acta* 538 (2005) 159.
- [28] J. Das, M.A. Aziz, H. Yang, *J. Am. Chem. Soc.* 128 (2006) 16022.
- [29] M. Han, X. Gao, J. Su, S. Nie, *Nat. Biotechnol.* 19 (2001) 631.
- [30] J. Wang, G. Liu, R. Polsky, *Electrochem. Commun.* 4 (2002) 722.
- [31] J. Wang, G. Liu, A. Merkoçi, *J. Am. Chem. Soc.* 125 (2003) 3214.
- [32] G. Liu, J. Wang, J. Kim, M.R. Jan, G.E. Collins, *Anal. Chem.* 76 (2004) 7126.
- [33] H. Wu, G. Liu, J. Wang, Y. Lin, *Electrochem. Commun.* 9 (2007) 1573.
- [34] J.A. Hansen, J. Wang, A. Kawde, Y. Xiang, K.V. Gothelf, G. Collins, *J. Am. Chem. Soc.* 126 (2006) 2228.
- [35] R. Thurer, T. Vigassy, M. Hirayama, J. Wang, E. Bakker, E. Pretsch, *Anal. Chem.* 79 (2007) 5107.
- [36] J. Wang, *Electroanalysis* 17 (2005) 7.
- [37] P. He, Y. Xu, Y. Fang, *Microchim. Acta* 152 (2006) 175.
- [38] K. Gong, Y. Yan, M. Zhang, L. Su, S. Xiong, L. Mao, *Anal. Sci.* 21 (2005) 1383.
- [39] M. Trojanowicz, *Trends Anal. Chem.* 25 (2006) 480.
- [40] Y. Lin, W. Yantasee, J. Wang, *Front. Biosci.* 10 (2005) 492.
- [41] J. Wang, G. Liu, M.R. Jan, *J. Am. Chem. Soc.* 126 (2004) 3010.
- [42] B. Munge, G. Liu, G. Collins, J. Wang, *Anal. Chem.* 76 (2004) 7126.
- [43] X. Yu, B. Munge, V. Patel, G. Jensen, A. Bhirde, J.D. Gong, S.N. Kim, J. Gillespie, J.S. Gutkind, F. Papadimitrakopoulos, J.F. Rusling, *J. Am. Chem. Soc.* 128 (2006) 11199.
- [44] G. Liu, J. Wang, Y. Lin, *ChemBiochem.* 7 (2006) 1315.
- [45] G. Liu, J. Wang, H. Wu, Y. Lin, *Anal. Chem.* 78 (2006) 7417.
- [46] (a) G. Liu, H. Wu, J. Wang, Y. Lin, *Small* 2 (2006) 1139;
(b) G. Liu, Y. Lin, *J. Am. Chem. Soc.* 129 (2007) 10394.
- [47] G. Liu, H. Wu, A. Dohnalkova, Y. Lin, *Anal. Chem.* 79 (2007) 5614.
- [48] G.C. Ford, P.M. Harrison, D.W. Rice, J.M.A. Smith, A. Treffry, Y.J. White, *Philos. Trans. R. Soc. Lond. Ser. B* 304 (1984) 551.
- [49] H.A.H. Rongen, A. Bult, W.P. Van Bennekom, *J. Immunol. Methods* 204 (1997) 105.
- [50] K.A. Edwards, A.J. Baeumner, *Talanta* 68 (2006) 1432.
- [51] K.A. Edwards, A.J. Baeumner, *Talanta* 68 (2006) 1421.
- [52] P. D'Orazio, G.A. Rechnitz, *Anal. Chem.* 49 (1977) 2083.
- [53] P. D'Orazio, G.A. Rechnitz, *Anal. Chim. Acta* 109 (1979) 25.
- [54] K. Shiba, Y. Wanstabe, S. Ogawa, S. Fujiwara, *Anal. Chem.* 52 (1980) 1610.
- [55] R.M. Kannuck, J.M. Bellama, *Anal. Chem.* 60 (1988) 142.
- [56] A.J. Baumner, R.D. Schmid, *Biosens. Bioelectron.* 13 (1998) 519.
- [57] K.S. Lee, T. Kim, M. Shin, W. Lee, J. Park, *Anal. Chim. Acta* 380 (1999) 17.
- [58] M. Haga, S. Sugawara, H. Itagaki, *Anal. Biochem.* 118 (1981) 286.
- [59] H. Chen, J. Jiang, Y. Li, T. Deng, G. Shen, R. Yu, *Biosens. Bioelectron.* 22 (2007) 993.
- [60] J.F. Chen, H.M. Ding, J.X. Wang, L. Shao, *Biomaterials* 25 (2004) 723.
- [61] W. Lian, S.A. Litherland, H. Badrane, W.H. Tan, D.H. Wu, H.V. Baker, P.A. Gulig, D.V. Lim, S.G. Jin, *Anal. Biochem.* 334 (2004) 135.
- [62] M. Qhobosheane, S. Santra, P. Zhang, W.H. Tan, *Analyst* 126 (2001) 1274.
- [63] I. Roy, T.Y. Ohulchanskyy, D.J. Bharali, H.E. Pudavar, R.A. Mistretta, N. Kaur, P.N. Prasad, *Proc. Natl. Acad. Sci. U.S.A.* 102 (2005) 279.
- [64] S. Santra, P. Zhang, K. Wang, R. Tapecc, W. Tan, *Anal. Chem.* 73 (2001) 4988.
- [65] J. Wang, G. Liu, Y. Lin, *Small* 2 (2006) 1134.
- [66] J. Wang, G. Liu, M.H. Engelhard, Y. Lin, *Anal. Chem.* 78 (2006) 6974.
- [67] J. Wang, G. Liu, B. Munge, L. Lin, Q. Zhu, *Angew. Chem. Int. Ed.* 43 (2004) 2158.
- [68] W.C. Mak, K.Y. Cheung, D. Trau, A. Warsinke, F. Scheller, R. Renneberg, *Anal. Chem.* 77 (2005) 2835.
- [69] J. Wang, G. Liu, G. Rivas, *Anal. Chem.* 75 (2003) 4661.
- [70] J. Wang, G. Liu, *Anal. Chem.* 78 (2006) 2461.
- [71] J. Wang, G. Liu, Q. Zhu, *Anal. Chem.* 75 (2003) 6218.
- [72] S.R. Nicewarner-Pena, R.G. Freeman, B.D. Reiss, L. He, D.J. Pena, I.D. Walton, R. Cromer, C.D. Keating, M.J. Natan, *Science* 294 (2001) 137.
- [73] J.B.-H. Tok, F.Y.S. Chuang, M.C. Kao, K.A. Rose, S.S. Pannu, M.Y. Sha, G. Chakarova, S.G. Penn, G.M. Dougherty, *Angew. Chem. Int. Ed.* 45 (2006) 6900.

Review

Chemical modification of polymeric microchip devices[☆]

Alexander Muck^{*}, Aleš Svatoš

*Mass Spectrometry Research Group, Max Planck Institute for Chemical Ecology,
Hans-Knöll-Straße 8, D-07745 Jena, Germany*

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Abstract

Analytical polymeric microchips in both fluidic and array formats offer short analysis times, coupling of many sample processing and chemical reaction steps on one platform with minimal sample and reagent consumption, as well as low cost, minimal fabrication times and disposability. However, the invariable bulk properties of most commercial polymers have driven researchers to develop new modification strategies. This article critically reviews the scope and development of chemical modifications of such polymeric chips since 2003. Surface modifications were based on chemical derivatization or activation of surface layers with reagent solutions, reactive gases and irradiation. Bulk modification of polymer chips used newly incorporation of monomers with selective chemical functionalities throughout the bulk polymer material and integrated the chip modification and fabrication into a single step. Such modifications hold a great promise for establishing a true ‘lab-on-chip’ as can be seen from many novel applications for modulating electroosmosis, suppressing protein adsorption in microchip capillary electrophoretic separations, extraction of analytes and for zone-specific binding of enzymes and other biomolecules.

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Keywords: Polymer; Chemical modification; Surface; Bulk; Array; Electroosmotic flow; Adsorption; Functionalization; Enzyme; Review

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Abbreviations: AEMA, aminoethyl methacrylate; APTES, (3-aminopropyl)-triethoxysilane; BMA, butyl methacrylate; BSA, bovine serum albumin; CA I, carbonic anhydrase; CLiPP, contact liquid photolithographic polymerization; COC, cyclic olefin copolymer; Cyt C, cytochrome C; DNA, deoxyribonucleic acid; EDS, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ELISA, enzyme-linked immunosorbent assay; EOF, electroosmotic flow; HHem, horse hemoglobin; IgG, immunoglobulin; IMAC, immobilized metal ion affinity chromatography; LYS, lysozyme; M, mol L⁻¹; MALDI-TOF/MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; MAA, methacrylic acid; MCE, microchip electrophoresis; MeCN, acetonitrile; MEKC, micellar electrokinetic chromatography; MMA, methyl methacrylate; Myo, horse myoglobin; PC, poly(carbonate); PDMS, poly(dimethylsiloxane); PEGAA, poly(ethyleneglycol diacrylate); pK_a, acidic dissociation constant; pMALDI, polymeric MALDI sample support array chip; PMMA, poly(methyl methacrylate); SEMA, sulfoethyl methacrylate; SPE, solid phase extraction; TRP, trypsinogen; UV, ultra violet; μ_{eo} , mobility of the electroosmotic flow; ζ , zeta potential.

[☆] This review article has been written on the occasion of Prof. Joseph Wang's 60th birthday.

^{*} Corresponding author. Tel.: +49 3641 571809; fax: +49 3641 571701.

E-mail address: amuck@ice.mpg.de (A. Muck).

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1. Introduction

The integration of many analytical operations on a miniaturized platform has been envisioned as the ‘lab-on-chip’ concept by Manz and Widmer in the last decade [1,2]. These efforts follow a similar miniaturization trend from the electronics industry of the seventies and eighties gaining major benefits from the powerful microfabrication techniques, semiconductor physics and from the use of novel materials. Such microchip systems allow for minimal sample and power consumption, enable sample pretreatment, various chemical reactions, fast separation and detection times. Since the emergence of the concept, numerous novel approaches have been published. Especially polymers have been recently studied for their lower cost, number of replication strategies and disposability [3,4] and currently their applications to new analytical areas are being extensively studied. These developments have been summarized in several general reviews [3–5].

Surface chemistry is of great importance when the large surface-to-volume ratio of analytical microchips is considered either in the microfluidic (utilizing miniaturized capillary electrophoretic or free-flow zone electrophoretic separation) or in the high-throughput array formats (as sample supports for solid phase extraction of analytes, as miniaturized bioassay platforms or miniaturized detections vessels) in combination with various means of detections (electrochemical, optical or mass spectrometric). In these devices, adsorption and surface effects play an increased role in comparison to classical capillary systems. Although these microchip systems have mostly been prepared from fused silica and glass substrate materials due to their good optical properties and chemical inertness, they are difficult to fabricate and only a limited range of modification strategies is known. Therefore, alternative materials such as thermoplastic and UV-curing polymers have been introduced in this scientific field [4].

Earlier polymer chip modifications have been summarized in good reviews on modification strategies for microchip electrophoresis (MCE) by Belder and Ludwig [6] and on modification of PDMS chips in MCE and bioassays by Makamba et al. [7]. The emergence of polymer chip modification as a research topic in analytical chemistry is well illustrated by the first contributions and increase of the publication activity in the early years of the 21st century reaching a certain maturation in the period of 2005–2006 (see Fig. 1) as illustrated by the publication number increasing from 3 in the year 2002 to 12 and 11 in the years 2003 and 2004 and remaining stable at similar numbers in the period 2005–2006 (using keywords ‘polymer’, ‘chip’ and ‘mod-

ification’ for search in the Web of Science databases). A similar less specific search (using the keywords ‘chip’ and ‘modification’) confirms this trend with 3, 6, 12, 14 and 23 publications dealing with modification of polymer or hybrid analytical chips in the years 2002–2006, respectively. The lack of new modification strategies could be attributed to a limited number of commercial polymer materials used for microfabrication of such devices and to the large number of modification strategies well established on these polymeric materials for immobilization of enzymes in enzyme-linked immunosorbent assays (ELISA) in the nineties. Due to such material limitations, we believe, that the modification of polymeric microchips should not be limited to surface modifications and that new polymeric substrates are urgently needed, enabling further fabrication and modification protocols.

While the modifications of polymer MCE chips were mainly driven by efforts to increase electroosmotic flow (EOF) velocity and thus improve the reproducibility of electrokinetic injections and of day-to-day separations or to reduce non-specific binding of bioanalytes on the walls of unmodified devices, for other systems – such as enzyme reactors or bioassay platforms – an efficient binding of reagents and bioprobes was essential. To achieve these goals, dynamic (non-permanent) and permanent coatings physically adsorbed to the surface and covalently bound permanent coatings have been studied. Our review describes the major contributions to such modifications of polymeric chips in the last 5 years with the main focus on new bulk covalent modification strategies and applicability of different modification strategies for real micro-total-analytical systems.

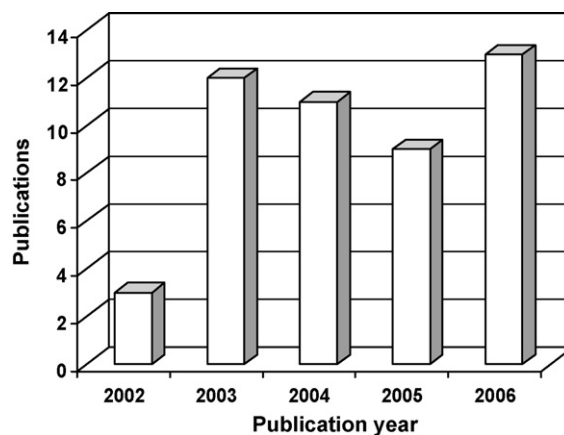


Fig. 1. Number of publications on modifications of polymer analytical chips in the period of 2002–2006 (complete years) available from Web of Science scientific databases using keywords ‘modification’ and ‘polymer’ and ‘chip’.

2. Modulation of the electroosmotic flow

The EOF is considered to be a non-selective additional driving force in electrophoretic separations influencing the separation efficiency (improving separation if its direction is opposite to the electrophoretic movement of the analytes and decreasing the separation in case of identical direction). Because of the low surface charge densities and thus lower surface zeta (ζ) potentials, the EOF in polymeric devices is much lower than for the glass-based fluidic chips. Therefore, many groups have been focusing on the modulation and increase of the ionic character of polymeric substrates used for fabrication of such separation chips or on surface activation of the microfluidic network itself [6], which also leads to a significant reduction of adsorption of more hydrophobic analytes to the polymeric surfaces (causing irreproducible EOF and loss of resolution).

2.1. PMMA

The patterned photochemical activation of PMMA microfluidic devices by UV light was in detail studied by Soper's group [8]. The authors demonstrated the relationships between the UV exposure time and the density of the generated carboxylic acids as well as the EOF, surface wettability and topography.

A new bulk modification/fabrication concept has been introduced for acrylate chips [9,10] using copolymerization of methyl methacrylate monomer (MMA) with modifying monomer molecules (e.g., methylacrylic acid (MAA), 2-sulfoethyl methacrylate (2-SEMA), 2-aminoethyl methacrylate (2-AEMA)) during the atmospheric-pressure molding fabrication protocol. The copolymerization of these monomers is UV-initiated, proceeds by free radical mechanism, and forms a complete graft poly(methyl methacrylate-co-alkyl methacrylate) polymer. This approach offered controlled loading of the modifying moieties to increase hydrophobicity or introduce ionizable functional groups. Thus, in-situ tailored migration properties of the resulting microchips were obtained within the chip fabrication itself (a general schematic of the copolymerization of methacrylates is shown in Fig. 2A). A selection of the functional group of the modifier can thus lead to anionic, neutral, and cationic surfaces, and can impart a wide range

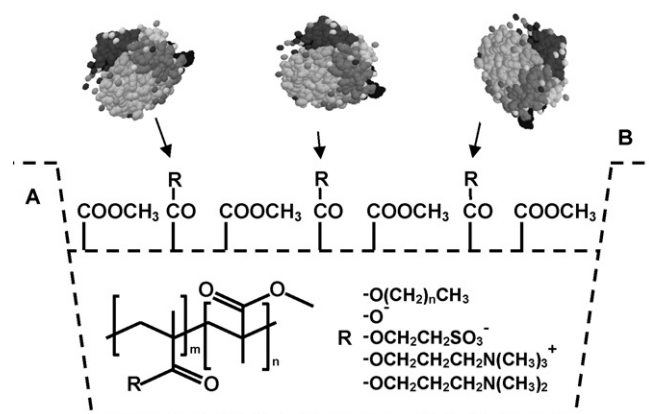


Fig. 2. (A) Atmospheric molding of modified copolymer chips with tailored hydrophobicity/ionic properties using different alkyl methacrylates (R–) and (B) solid phase extraction of proteins on a polymer MALDI-TOF chip sample zone for integrated protein preconcentration and spectral acquisition (reprinted with permission [19]).

of transport properties. Moreover, the need for an additional activation or modification process is obviated. Thus, such fabrication could further reduce the time and production costs of plastic chips. The manipulation of the bulk material was used for significant enhancement or reversal of the electroosmotic flow mobility (μ_{eo}) [9]. Whereas the low μ_{eo} values of the bare PMMA (Fig. 3A,a) were almost unaltered by the change of pH, a significant increase of the μ_{eo} has been achieved by a 6% MAA incorporation into the polymer substrate (Fig. 3A,b) which could at pH 6 reach that of a glass microchip (Fig. 3A,c). The μ_{eo} of both the bulk-modified and unmodified substrates exhibited cathodic flows, indicating excess of negative charge of the microchip material. However, compared to the plain PMMA chip where the μ_{eo} was only slightly affected by the increasing pH ($1.80\text{--}2.4\text{ cm}^2\text{ V}^{-1}\text{ s}^{-1}$ over the pH 3.5–9.5 range), the MAA modified microchip displayed a strong pH sensitivity (with higher EOF at higher pH values). This dependence showed a pronounced sigmoidal shape with an inflex above the pK_a of the carboxylic acid (4.66). Such profile indicated the accessibility on the surface carboxylic groups and full deprotonation above pH 6, leading to a maximum surface charge density. A reversal of the EOF was observed using a 2-aminoethyl-methacrylate modi-

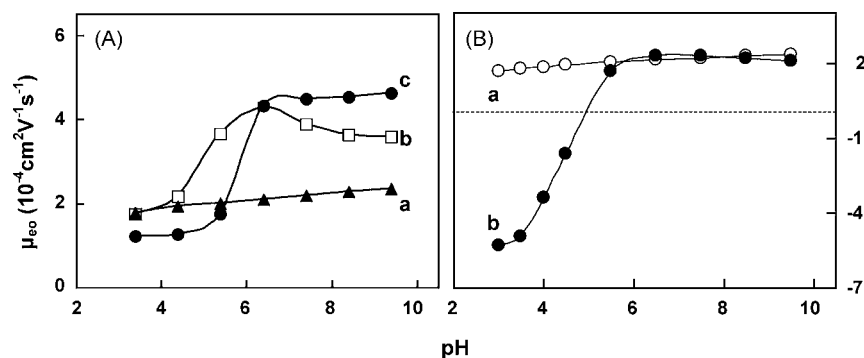


Fig. 3. Manipulation of the electroosmotic flow by bulk modification of polymeric chip materials. (A) Increase of the electroosmotic flow mobilities (μ_{eo}) of the unactivated bare PMMA chip (a) by a 6% MAA bulk modification (b) compared to a glass microchip (c), and (B) reversal of the μ_{eo} of an unmodified PMMA microfluidic chip (a) by incorporation of 2% 2-AEMA (b) at different pH values (reprinted with permission [9]).

fied microchip (Fig. 3B,b). As expected for amine protonation at low pH values, the μ_{eo} became anodic below pH 5.0, reaching a value of $-5.6 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at pH 3.0; whereas above pH 6.5 the EOF direction was cathodic due to some free carboxylic groups and similar to that of the native PMMA (Fig. 3B,a).

A similar reversal of the electroosmotic flow has been done by a one step covalent immobilization of poly(ethyleneimine) onto PMMA substrates for MCE separation of basic proteins [11]. A further interesting approach to modulation and improvement of EOF for PMMA channels has been accomplished by a water-plasma hydroxylation of the PMMA side chains and subsequent reaction with 11-bromoundecyltrichlorosilane. The EOF μ_{eo} has been thus rendered stable and increased to $3.4 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ [12].

2.2. PDMS

Poly(dimethylsiloxane) (PDMS) is one of the frequently used materials in microchip capillary electrophoresis and microfluidics due to low cost and casting and polymerization at normal temperature, good attachment to glass and other polymeric materials, and elastomeric properties. The hydrophobicity of PDMS constraints its applicability for microchip electrophoretic systems and further non-electrophoretic applications; however, PDMS substrates can be modified [7] to increase their hydrophilicity and EOF using various plasma treatments, where silica gel-like structures are obtained. Attention should be paid to the short lifetime of such oxidized surfaces, which is decreased by a diffusion of unreacted oligomers to the surface. The oxidized surface lifetime could be increased by a two step method, where extraction of such unreacted monomers by a series of organic solvents has been performed before the plasma oxidation. Thus, hydrophilic Si-OH surfaces were stable for over 7 days and the EOF could be increased from 4.1 to $6.8 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ [13].

The control and enhancement of the EOF in PDMS channels could also be accomplished by a copolymerization of 0.5% wt. undecylenic acid with the prepolymer solution. The μ_{eo} has been increased to $7.6 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ which was nearly twice higher than that of the unmodified polymer and allowed for additional polysaccharide dynamic coating for suppression of non-specific adsorption [14].

2.3. Other polymers

A UV treatment of cyclic olefin copolymer (COC) fluidic chips has reduced the contact angle of water almost twice from 88° to 45° . Such simple modification could improve the liquid handling and capillary driven loading of the sieving polymer into the microchannels for a MCE separation of nucleic acids [15]. Similarly, a UV treatment has been used to activate surfaces of polycarbonate (PC) fluidic chips for electrokinetic sorting of *Escherichia coli* and *Saccharomyces cerevisiae* cells by Witek et al. [16]. A notable comparison of physicochemical properties of various polymer (PMMA, PC, polyethylene polyterephthalate, polypropylene, polysulfone, polyurethane, etc.) substrates used for hot embossing of microfluidic devices has been provided

by Shadpour et al. The authors described glass temperatures, replication accuracies and EOF mobilities of these substrates for myosin in both the untreated and the BSA passivated form [17].

A new fabrication technique ‘contact liquid photolithographic polymerization’ (CLiPP) has enabled further useful modification route to many custom-designed polymeric substrates [18]. This technique combined the principles of silicon micromachining of microfluidic components with the polymer molding techniques by enabling development of three-dimensional copolymeric devices and incorporation of a living radical initiator (the ‘photoinferter’ precursor containing a dithiocarbamate group) to monomer mixtures (acrylic acid, 1-hydroxycyclohexyl phenyl ketone, triethyleneglycol diacrylate, etc.). Such photoinferter group enabled subsequent covalent bonding and surface grafting of the polymer layers and components.

3. Solid phase extraction

The dramatic increase of proteomic research in the recent years has been closely coupled to development of new analytical approaches and mass spectrometric instrumentation. As most protein identifications are performed by mass spectral measurements after several sample purification and pretreatment steps, these procedures can considerably increase the analysis costs as well as influence the precision and sensitivity of the measurements. Simplification of such sample pretreatment protocols has therefore been of high importance.

3.1. PMMA

Poly(butyl methacrylate-co-methyl methacrylate) pMALDI chips were introduced for solid phase extraction and MALDI-TOF/MS measurements of proteins in samples with high salt content by Svatoš's group [10] as a contribution to the current development of “gel-free” proteomic methods. Fig. 4 shows the MALDI-TOF/MS spectra of 10 pmol/ μL HHM sample containing 4 M urea after a simple one step desalting process (Fig. 4a). For sample without desalting, only a small peak was observed (Fig. 4b). Such sample pretreatments on plain PMMA or stainless steel surfaces resulted in a negligible degree of protein adsorption (Fig. 4c and e, respectively) and in no signals on these conventional surfaces without desalting (Fig. 4d and f, respectively).

The in-situ bulk modification approach has been also tested for pH selective SPE preconcentration and on-chip MALDI-TOF/MS protein measurements by Muck et al. [19]. Several monomers containing anionic and cationic alkyl methacrylate monomers, such as methacrylic acid (MAA), 2-sulfoethyl methacrylate (SEMA), and 3-(*N,N*-dimethylaminopropyl) methacrylate (DMAPMA) have been copolymerized with methyl methacrylate (MMA) backbone to yield a specific strong/weak ionic surface charge of the pMALDI substrates. It was shown, that the negatively charged sulfo functionalities from the strongly anionic poly(2-sulfoethyl methacrylate-co-methyl methacrylate) copolymer

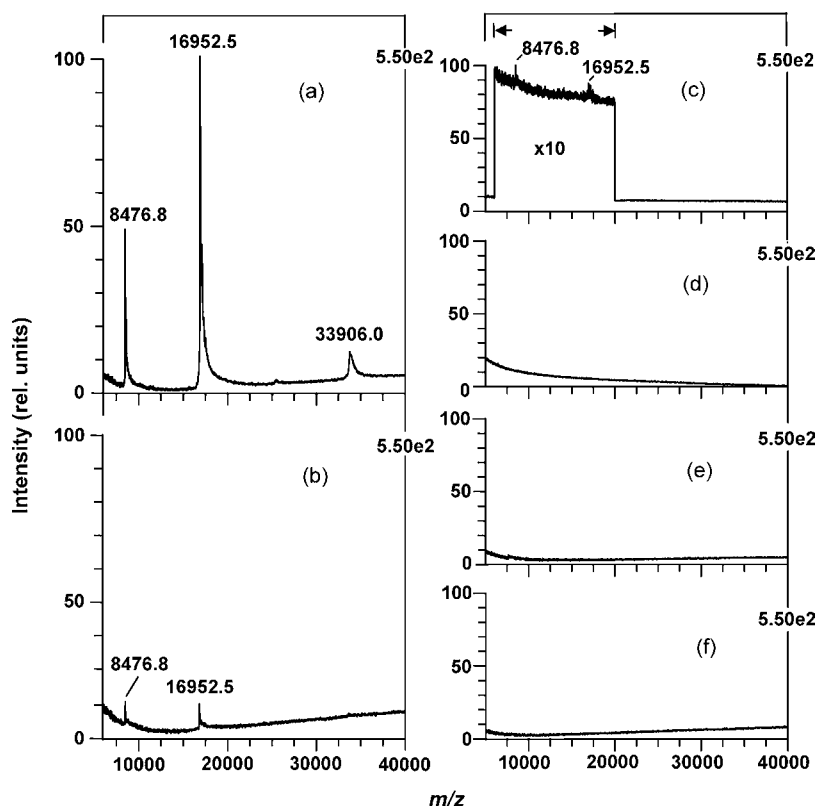


Fig. 4. Enhanced desalting/solid phase extraction of a protein sample on a pMALDI chip with increased hydrophobicity: MALDI-TOF spectra of horse heart myoglobin (10 pmol) in 4 M urea and 0.1% trifluoroacetic acid after on-target desalting (a) on a hydrophobic poly(butyl methacrylate-co-methyl methacrylate) pMALDI chip (monomer ratio 8/2, v/v), unmodified PMMA (c) and steel (e) targets, compared to spectra recorded on the poly(butyl methacrylate-co-methyl methacrylate) (b), PMMA (d) and steel (f) devices without desalting (reprinted with permission [10]).

(SEMA/MMA) pMALDI chip could thus attract oppositely charged groups of protein chains from protein mixtures based on the protein *pI* and the pH of the chosen buffer (Figs. 2B and 5). The non-specific binding and signals of unbound proteins on the polymer surface were counterbalanced by a post-adsorption washing step with a protein-free solution; making the MALDI-TOF/MS signals mainly attributable to proteins which were strongly bound to the chip surface. After increasing the pH of the protein solution stepwise from 4 to 6 (Fig. 5b) and washing with the identical buffer, distinct adsorption profiles and selective preconcentration of lysozyme (LYS), trypsinogen (TRP), and β -lactoglobulin (β LG) according to their *pI* values (11, 9 and 5, respectively) were observed on the negatively charged chip. By further increasing the solution pH to 8, the adsorption of β LG was strongly suppressed (Fig. 5c); at pH value 10 (Fig. 5d) only LYS (*pI* 11) showed adsorption to the surface.

A buffer-ion permeable copolymeric membrane has been fabricated by Liu et al. [20] between two parallel channels of a poly(glycidyl methacrylate-co-methyl methacrylate) copolymeric chip after activation of the surface with ethylenediamine to provide reactive amine groups for further attachment of isocyanatoethyl methacrylate and further crosslinking/polymerization with a mixture of ethylene glycol acrylate with methyl methacrylate in a Tris-Cl buffer. A 400-fold upconcentration of green fluorescent protein has been thus achieved using the micro electric field gradient focusing.

3.2. Other polymers

An earlier in situ fabrication approach for preparation of copolymeric chemically functionalized membranes by a polycondensation reaction from non-miscible aqueous and organic solutions has been described by Kitamori's group. A two-phase flow has been used in X-shaped microchannels on aminopropyl-triethoxysilylated surfaces of glass chips for polycondensation of adipoyl chloride and hexamethylenediamine to nylon 6-6. These elastic membranes could be used for immobilization of horseradish peroxidase and testing of enzymatic reactions based on permeation of substrates from separated microchannel compartments [21]. A simple UV photoactivation of PC channels containing miniature adsorption posts has been applied for adsorption and purification of dye labeled DNA sequencing fragments by a solid phase reversible immobilization prior to their gel electrophoretic separation [22].

4. Suppression of protein adsorption/electrophoretic separations

Adsorption of biopolymers such as proteins on channel walls is a common problem for microfluidic devices fabricated from any conventional material, including organic polymers, resulting in sample and performance loss. Therefore, the surfaces have to be passivated to minimize the non-specific

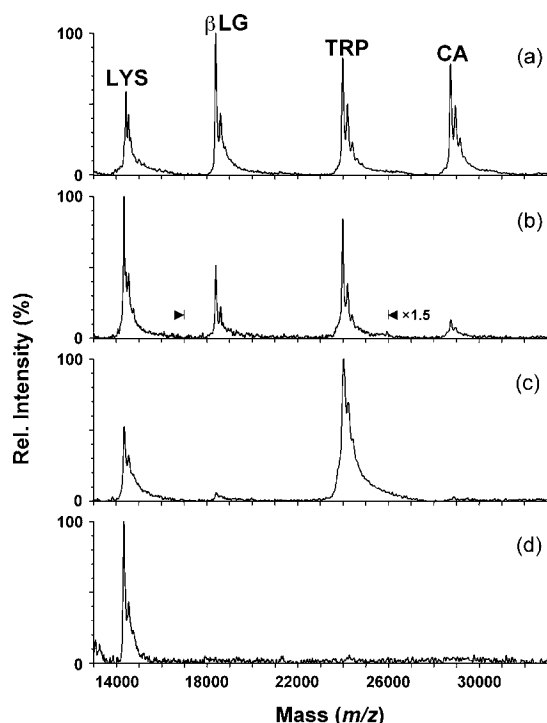


Fig. 5. Selective adsorption of lysozyme (LYS), β -lactoglobulin (β LG), trypsinogen (TRP), and carbonic anhydrase (CA I) mixture (5, 35, 50, and 5 pmol, respectively) on an anionic SEMA/MMA chip. Mass spectral protein profile after deposition of the complete sample solution (a), compared to mass spectra of the mixture adsorbed at pH 6, 8, and 10 (b, c, and d, respectively) (reprinted with permission [19]).

adsorptions. Mainly two approaches have been used, dynamic coatings and permanent surface modifications. However, the permanent surface modification requires tedious multistep physical/chemical processing, which complicates the preparation of such polymeric microfluidic chips. Development of new polymeric materials, possessing inherent protein resistance and good physical properties which could be used to fabricate microfluidic devices without further surface modification was therefore of great interest [23].

4.1. PMMA

A dynamic coating of PMMA channels by poly(ethyleneoxide) (PEO) has been used by Lin et al. to minimize the interaction of DNA with the polymer surface during MCE. Different dynamic coatings were tested including charged cellulose derivatives, such as 2-hydroxyethyl cellulose, hydroxypropyl cellulose and polyvinyl pyrrolidone (PVP). Highest electrophoretic separation efficiencies were finally obtained by a multilayer coating with PEO, PVP and PEO containing gold nanoparticles [24].

A new modification and bonding technique 'resin-gas injection' has been introduced by Lai et al. for PMMA [25]. In this technique, an efficient channel bonding was achieved by infusing a dilute solution of UV sensitive hydroxyethyl methacrylate by nitrogen pumping, by subsequent blowing out of the monomer from the microchannels to leave only a thin layer on the surfaces and by UV initiated polymerization. Thus, copoly-

mers of poly(hydroxyethyl methacrylate-co-ethylene glycol dimethacrylate) could be immobilized on the internal walls of the channels forming a monolithic stationary gel for an acrylamide separation of DNA.

Suppression of the non-specific adsorption of bovine serum albumin and enhancement of the hydrophilic properties of PMMA chips by surface grafting of different hydrophilic monomers has been studied in detail by Stachowiak et al. [26]. The surface has been rendered hydrophilic in defined zones by UV irradiation of monomer solutions in the microchannels with surface bound photoinitiator molecules through optical masks. The protein adsorption has thus been suppressed to less than 2% relative to the pristine surface. Another dynamic approach to altering the surface charge of PMMA substrate and suppression of protein adsorption to the separation channels, by a mixture of cetyltrimethylammonium bromide with sodium dodecyl sulfate (SDS) has been used for micellar electrokinetic chromatographic separation (MEKC) of lipoproteins [27].

A new application of the atmospheric molding technique [28] has been developed by Liu et al. [23] to test protein and peptide adsorption resistant copolymeric chips by copolymerizing poly(ethyleneglycol diacrylate) (PEGAA) and MMA in a sandwich mold containing a silicon master with the microchannel negative structure. A similar chemistry has been chosen by Bi et al. to form brush like adsorbed structures of poly(ethylene glycol dimethacrylate-co-methylether methacrylate) copolymer on the surface of PMMA chips for increasing the hydrophilicity and electrophoretic separation of proteins [29].

First attempts to introduce a biomimetic (biocompatible) surface on a PMMA chip have been made by hydrophobic attachment of butyl moieties of a phospholipid copolymer poly(2-methacryloyloxyethyl phosphorylcholine-co-butyl methacrylate) to the PMMA microchannel surface [30]. These chips exhibited stable electroosmotic flows and low adsorption of serum proteins. A hybrid dynamic coating was used in the same year to suppress the EOF and adsorption of analytes by flushing the PMMA electrophoretic chip channels by solutions of 0.25% of *n*-dodecyl β -D-maltoside and 0.03% methyl cellulose for analysis of N-linked glycans derived from glycosylated proteins by Baba's group [31].

4.2. PDMS

Oxygen plasma treatment and dynamic coating with heat-immobilized poly(vinyl alcohol) was used to render the hydrophobic PDMS surface hydrophilic and to suppress the EOF in a wide pH range 3–11 for obtaining high numbers of separation plates for separation of fluorescently labeled standard proteins and DNA fragments [32]. A covalent hydrophilic polymer coating on PDMS electrophoretic chips for suppression of lysozyme and bovine serum albumin adsorption during MEKC separation of proteins has been created by crosslinking epoxy-modified hydrophilic polymers onto an O_2 plasma pretreated surface following an aminopropyl silanization of the channels [33]. Finally, a simple modification of a PDMS substrate to increase the hydrophobicity by adsorbing a Teflon solution on a microfluidic channel outlet has been recently used to generate

hydrodynamic droplets for bioanalytical dispensing coupled to bioseparation chips [34].

5. Biochips/arrays

To fulfill the growing needs for higher specificity of polymer analytical chips, the further modification strategies involved development of polymer-based protein or DNA planar chips (microarrays) by binding biospecific probes such as enzymes, antibodies or nucleic acids to linker modified chip surfaces. A considerable research has also been done on coatings of standard glass supports by special polymers to increase the immobilization capacity and suppress non-specific binding [35]. This trend clearly suggests a wider usage of fully plastic chips for such bioprobe arrays.

5.1. PMMA

A reduction of the methyl ester groups on PMMA surfaces to hydroxymethyl functionalities by lithium aluminium hydride has been suggested for general surface modification of PMMA substrates by silylation reagents for protein or DNA arrays by Cheng et al. [36]. This protocol has been combined with direct-write laser micromachining and thermal annealing for smoothness improvement.

An integrated proteomic sample preparation/measurement pMALDI-TOF/MS chip has been fabricated by Ibáñez et al. using the atmospheric molding protocol/bulk modification strategy [37] and adding trypsin to the polymeric chip surface by covalently binding ethylene glycol disuccinate bis(sulfo-*N*-succinimidyl) ester with amine functionalities of the chip well surface and subsequent reaction of the linker sulfo group with 1.3 nmol trypsin (Fig. 6). The favorable performance of the new chips was demonstrated for the enzymatic digestion of individual proteins of 5–60 kDa size. A mixture of 500 fmol horse cytochrome C, bovine serum albumin, human hemoglobin, and horse myoglobin was deposited in the trypsin-linked sam-

ple well, followed by 15–60 min of on-chip digestion in a humidified chamber. Subsequent peptide mass fingerprinting using protein-database-searching software identified all four proteins. The combination of adsorption/signal enhancement of proteins on hydrophobic pMALDI arrays with the novel enzyme-linked chips is expected to minimize sample-handling times and enhance the analytical information in combination with the different scanning modes of modern MALDI-TOF instruments (offering intact protein mass measurements in the linear mode (Fig. 7A) combined with enzymatic cleavage and peptide mass fingerprint identification of the proteins in reflectron mode (Fig. 7B). This concept is being readily extended to high-throughput enzyme activity screening and protein processing. Recently, phosphorylated proteins and peptides were pre-concentrated on an IMAC-type PMMA chip using Ga(III) and Ni(II) metal ions and subsequently detected using a MALDI-TOF/MS instrument [38].

An enzymatic flow-through on-chip microreactor has been prepared by adsorbing trypsin on silanized PMMA surfaces [39] and later improved by immobilizing the enzyme on silica nanoparticles covalently bound to silanized PMMA substrates [40]. The enzymatic cleavage of proteins took place in less than 5 s and was also analyzed by MALDI-TOF/MS.

A dramatic decrease of necessary hybridization times for the detection of low abundant point mutations in colorectal cancer related genes has been demonstrated on simple flow through photomodified PMMA or PC/PDMS/PMMA hybrid chips by Soper's group [41,42]. The surface immobilized amine terminated oligonucleotide probes were deposited as arrays of detection spots in a microfluidic channel and could hybridize in less than 1 min (vs. 3 h required for conventional hybridization) to the presented mutated nucleic acids.

5.2. PDMS

Chemical surface modification methods of PDMS substrates have been also investigated for probe immobilization

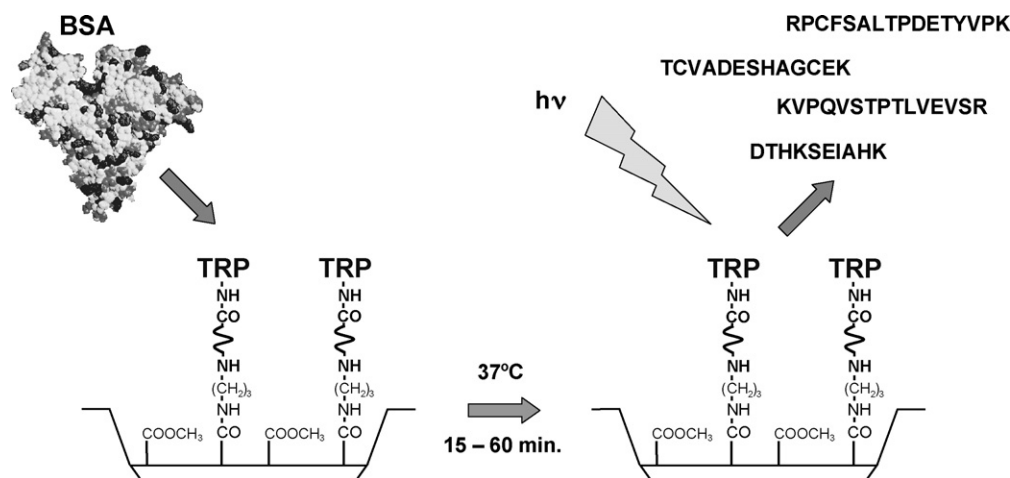


Fig. 6. On-chip protein digestion/identification using trypsin linked to an activated 2-aminoethyl methylacrylamide pMALDI chip surface via an polyethylene glycol linker. After short incubation period, matrix is added and a MALDI-TOF/MS acquisition is performed allowing for protein mass fingerprint identification (reprinted with permission [37]).

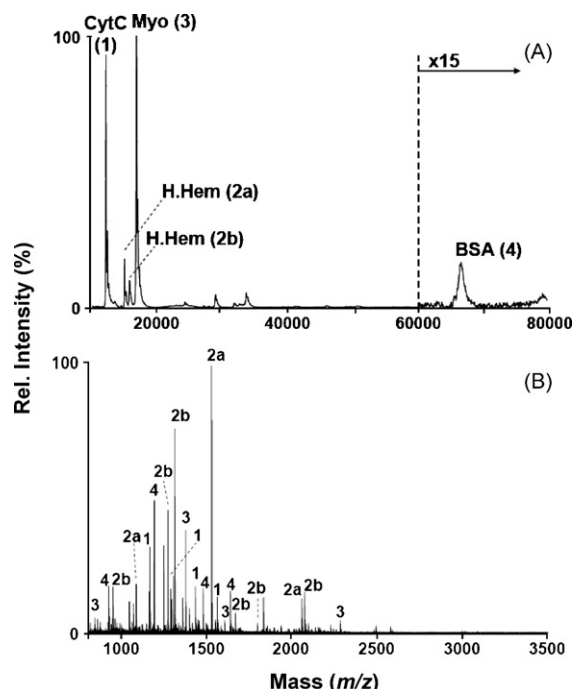


Fig. 7. Combining different modifications for efficient protein identification: (A) MALDI-TOF/MS linear mode spectrum of 0.5 pmol mixture of cytochrome C (Cyt C, 1), horse heart hemoglobin (H.Hem, 2), horse muscle myoglobin (Myo, 3), and bovine serum albumin (BSA, 4), on a BMA:MMA pMALDI chip, and (B) reflectron mode spectrum of the resulting peptide mixture (marked with protein labels) after on-chip digestion. The proteins were desorbed from the BMA:MMA chip using 30% MeCN/25 mM NaHCO₃ (pH 7.8) (reprinted with permission [37]).

in enzyme-linked immunosorbent assays (ELISA). The surfaces were silylated with amine containing (3-aminopropyl)-triethoxysilane (APTES) which were then either directly reacted with a glutaraldehyde crosslinker and the IgG of various organisms, or in a two step modification reacted with carboxyl groups of succinic acid anhydride followed by an 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDS), which could then capture the proteins. Such procedure coupled with competitive inhibition immunoassay yielded sensitivities in the pg/ml range [43].

5.3. Other polymers

Immobilization of DNA and proteins requires mostly reactive polymers. Thus, most of the applied materials contained epoxy, aldehyde or amine functionalities for biomolecule binding. Photogenerated reactive isothiocyanates in photosensitive poly(styrene-co-4-vinylbenzyl thiocyanate) have been employed for the reaction with amine-terminated oligonucleotides, providing good signal-to-noise ratios and reproducibility [35]. Reactive ion etching of cyclic olefin copolymers has been used for protein microarray patterning by Sohn et al. Patterns on COC surfaces have been modified by O₂, N₂ and CF₄ gas plasmas for improved immunoglobulin adsorption and compared to the use of other suitable polymeric microarray materials, such as PC and PMMA [44].

6. Conclusions

A short overview of the new approaches to modifications of poly(methyl methacrylate), poly(dimethylsiloxane), polycarbonate and cyclic olefin polymer chips was given. It has been shown, that due to constant material properties of most thermoplastic and elastomeric materials it is necessary to perform various chemical modifications to enable integration of different analytical operation steps on such disposable platforms. Covalent modification has proven to be very stable for most polymeric materials, whereas only few polymers were prone to loss of stability and coating with chemically and physically stable layers was used. Generally, functionalization of inherently hydrophobic microchip substrates with ionizable moieties has been used to increase the wettability and compatibility with most aqueous buffer systems, for manipulation and increase of separation efficiency in microchip electrophoresis, to induce pH-specific coulombic interaction with analytes enabling solid phase extractions, or to suppress non-specific adsorption of proteins from complex samples. It should be noted, that most inherent material properties (such as hydrophobicity) could be manipulated in different directions to reach different analytical goals (e.g. the increase of bioanalyte adsorption and removal of buffer components from complex mixtures on highly hydrophobic arrays). The new modification strategies including binding of bioprobes like enzymes or nucleic acids to microchip surfaces and research on new monomers with desirable properties is expected to enable the maturation of the current microchip chemistry and the establishment of real ‘laboratories-on-chip’ in the near future.

Congratulations

A.M. congratulates Prof. Joseph Wang to his 60th birthday and acknowledges gratefully his former supervision and professional expertise. Prof. Wang’s laboratory has been a crystallizing center of many great scientific ideas and a starting point for young scientists.



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References

- [1] D.J. Harrison, A. Manz, Z. Fan, H. Luedi, H.M. Widmer, Anal. Chem. 64 (1992) 1926.

- [2] D.J. Harrison, K. Fluri, K. Seiler, Z. Fan, C.S. Effenhauser, A. Manz, *Science* 267 (1993) 895.
- [3] D.R. Reyes, D. Iossifidis, P.A. Auroux, A. Manz, *Anal. Chem.* 74 (2002) 2623.
- [4] H. Becker, C. Gärtner, *Electrophoresis* 21 (2000) 12.
- [5] A. de Mello, *Lab Chip* 2 (2002) 31N.
- [6] D. Belder, M. Ludwig, *Electrophoresis* 24 (2003) 3595.
- [7] H. Makamba, J.H. Kim, K. Lim, N. Park, J.H. Hahn, *Electrophoresis* 24 (2003) 3607.
- [8] Y.S. Wei, B. Vaidya, A.B. Patel, S.A. Soper, R.L. McCarley, *J. Phys. Chem. B* 109 (2005) 16988.
- [9] J. Wang, A. Muck, M. Chatrathi, G. Chen, N. Mittal, S.D. Spillman, S. Obeidat, *Lab Chip* 5 (2005) 226.
- [10] A. Muck, P. Nešněrová, I. Pichová, A. Svatoš, *Electrophoresis* 26 (2005) 2835.
- [11] F. Kitagawa, K. Kubota, K. Sueyoshi, K. Otsuka, *Sci. Technol. Adv. Mater.* 7 (2006) 558.
- [12] T.M. Long, S. Prakash, M.A. Shannon, J.S. Moore, *Langmuir* 22 (2006) 4104.
- [13] J.A. Vickers, M.M. Caulum, C.S. Henry, *Anal. Chem.* 78 (2006) 7446.
- [14] Y.Q. Luo, B. Huang, H. Wu, R.N. Zare, *Anal. Chem.* 78 (2006) 4588.
- [15] R. Lin, M.A. Burns, *J. Micromech. Microeng.* 15 (2005) 2165.
- [16] M.A. Witek, S.Y. Wei, B. Vaidya, A.A. Adams, L. Zhu, W. Stryjewski, R.L. McCarley, S.A. Soper, *Lab Chip* 4 (2004) 464.
- [17] H. Shadpour, H. Musyimi, J. Chen, S.A. Soper, *J. Chromatogr. A* 1111 (2006) 238.
- [18] J.B. Hutchison, K.T. Haraldsson, B.T. Good, R.P. Sebra, N. Luo, K.S. Anseth, C.N. Bowman, *Lab Chip* 4 (2004) 658.
- [19] A. Muck, A.J. Ibáñez, E.J. Stauber, M. Mansourova, A. Svatoš, *Electrophoresis* 27 (2006) 4952.
- [20] J.K. Liu, X.F. Sun, P.B. Farnsworth, M.L. Lee, *Anal. Chem.* 78 (2006) 4654.
- [21] H. Hisamoto, Y. Shimizu, K. Uchiyama, M. Tokeshi, Y. Kikutani, A. Hibara, T. Kitamori, *Anal. Chem.* 75 (2003) 350.
- [22] Y.C. Xu, B. Vaidya, A.B. Patel, S.M. Ford, R.L. McCarley, S.A. Soper, *Anal. Chem.* 75 (2003) 2975.
- [23] J. Liu, X. Sun, M.L. Lee, *Anal. Chem.* 79 (2007) 1926.
- [24] Y.W. Lin, H.T. Chang, *J. Chromatogr. A* 1073 (2005) 191.
- [25] S.Y. Lai, X. Cao, L.J. Lee, *Anal. Chem.* 76 (2004) 1175.
- [26] T.B. Stachowiak, F. Švec, J.M.J. Fréchet, *Chem. Mater.* 18 (2006) 5950.
- [27] G.C. Ping, B.M. Zhu, M. Jabasini, F. Xu, H. Oka, H. Sugihara, Y. Baba, *Anal. Chem.* 77 (2005) 7282.
- [28] A. Muck, J. Wang, M. Jacobs, G. Chen, M.P. Chatrathi, V. Jurka, Z. Výborný, M.J. Schöning, *Anal. Chem.* 76 (2004) 2290.
- [29] H. Bi, S. Meng, Y. Li, K. Guo, Y. Chen, J. Kong, P. Yang, W. Zhong, B. Liu, *Lab Chip* 6 (2006) 769.
- [30] H. Bi, W. Zhong, S. Meng, J. Kong, P. Yang, B. Liu, *Anal. Chem.* 78 (2006) 3399.
- [31] F.Q. Dang, K. Kakehi, J.J. Cheng, O. Tabata, M. Kurokawa, K. Nakajima, M. Ishikawa, Y. Baba, *Anal. Chem.* 78 (2006) 1452.
- [32] D.P. Wu, Y. Luo, X.M. Zhou, Z.P. Dai, B.C. Dai, *Electrophoresis* 26 (2005) 211.
- [33] D. Wu, B. Zhao, Z. Dai, J. Qin, B. Lin, *Lab Chip* 6 (2006) 942.
- [34] S.J. Kim, Y.A. Sung, P.L. Skipper, J. Han, *Anal. Chem.* 78 (2006) 8011.
- [35] C. Preininger, U. Sauer, W. Kern, J. Dayteg, *Anal. Chem.* 76 (2004) 6130.
- [36] J.Y. Cheng, C.W. Wei, K.H. Hsu, T.H. Jang, *Sens. Actuators B* 99 (2004) 186.
- [37] A.J. Ibáñez, A. Muck, V. Halim, A. Svatoš, *J. Prot. Res.* 6 (2007) 1183.
- [38] A.J. Ibáñez, A. Muck, A. Svatoš, *J. Prot. Res.* 6 (2007) 3842.
- [39] H. Qu, H. Wang, Y. Huang, W. Zhong, H. Lu, J. Kong, P. Yang, B. Liu, *Anal. Chem.* 76 (2004) 6426.
- [40] Y. Huang, W. Shan, B. Liu, Y. Liu, Y. Zhang, Y. Zhao, H. Lu, Y. Tang, P. Yang, *Lab Chip* 6 (2006) 534.
- [41] Y. Wang, B. Vaidya, H.D. Fahrquar, W. Stryjewski, R.P. Hammer, R.L. McCarley, S.A. Soper, Y.W. Cheng, F. Barany, *Anal. Chem.* 75 (2003) 1130.
- [42] S.A. Soper, M. Hashimoto, C. Situma, M.C. Murphy, R.L. McCarley, Y.W. Cheng, F. Barany, *Methods* 37 (2005) 103.
- [43] L. Yu, C.M. Li, Q. Zhou, *Front. Biosci.* 10 (2005) 2848.
- [44] Y.S. Sohn, J. Kai, C.H. Ahn, *Sens. Lett.* 2 (2004) 171.

Review

Magnetic beads as versatile tools for electrochemical DNA and protein biosensing

Emil Paleček*, Miroslav Fojta

Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i, Královopolská 135, CZ-612 65 Brno, Czech Republic

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On the occasion of the 60th birthday of Professor Joseph Wang.

Abstract

Magnetic beads (MBs) are versatile tools in the separation of nucleic acids, proteins and other biomacromolecules, their complexes and cells. In this article recent application of MBs in electrochemical biosensing and particularly in the development of DNA hybridization sensors is reviewed. In these sensors MBs serve not only for separation but also as a platform for optimized DNA hybridization. A hybridization event is detected separately at another surface, which is an electrode. The detection is based either on the intrinsic DNA electroactivity or on various kinds of DNA labeling, including chemical modification, enzyme tags, nanoparticles, electroactive beads, etc., greatly amplifying the signals measured. In addition to DNA hybridization, other kinds of biosensing in combination with MBs, such as DNA–protein interactions, are reviewed.

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Keywords: Magnetic beads; Biosensing; DNA hybridization; DNA–protein interactions; DNA labeling; Nanoparticles; Enzymes; Stripping; Electrochemical analysis

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Abbreviations: A, adenine; ALP, alkaline phosphatase; C, cytosine; CNTs, carbon nanotubes; CPS, constant current chronopotentiometry; CPSP, constant current chronopotentiometric stripping analysis; DE, detection electrode; DPP, differential pulse polarography; dsDNA, double-stranded DNA; DST, double-surface technique; EP, Emil Paleček; G, guanine; HRP, horse radish peroxidase; JW, Joseph Wang; LSW, linear sweep voltammetry; MB, magnetic beads; MutS, MutS protein; NA's, nucleic acids; ODN, oligodeoxynucleotide; Os(VI)L, osmium (VI) complexes; Os(VIII)L, osmium tetroxide complexes with nitrogen ligands; PNA, peptide nucleic acid; RP's, signaling (reporter) ODN probes; SCE, saturated calomel electrode; ssDNA, single-stranded DNA; T, thymine; tDNA, target DNA; U, uracil.

* Corresponding author at: Laboratory of Biophysical Chemistry and Molecular Oncology, Institute of Biophysics, Academy of Sciences of the Czech Republic, Kralovopolska 135, CZ-612 65 Brno, Czech Republic. Tel.: +420 5 49246 241; fax: +420 5 415 17 249.

E-mail address: palecek@ibp.cz (E. Paleček).

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1. Introduction

Renaturation and hybridization of DNA, i.e. its ability to reform the DNA double-helical structure from the complementary single strands [1,2], was discovered by J. Marmur (JM) more than 45 years ago. This ability of DNA is utilized in a number of molecular biological methods and in modern biotechnologies, including the DNA electrochemical sensors. JM also proposed a method of DNA isolation, which was used, with some modifications, for decades as a general method of DNA isolation from bacteria and other organisms [3]. His paper was quoted >9000 times according to the Web of Science. This method represented a great improvement of the procedures earlier used for DNA isolation; however, one of us (EP, who worked in JM's lab as a postdoc), recalls a complex series of precipitation and washing steps which were time-consuming and laborious to perform; working from the morning till hours after midnight was not exceptional.

At present alternative methods, based on DNA binding to solid surface offering faster and less time-consuming procedures, are available. Among them magnetic separation has gained renewed interest over the last decade because of its advantages over traditional nonmagnetic methods. This method uses magnetism for efficient separation of cells, cell organelles as

represented a great improvement of the procedures earlier used for DNA isolation; however, one of us (EP, who worked in JM's lab as a postdoc), recalls a complex series of precipitation and washing steps which were time-consuming and laborious to perform; working from the morning till hours after midnight was not exceptional.

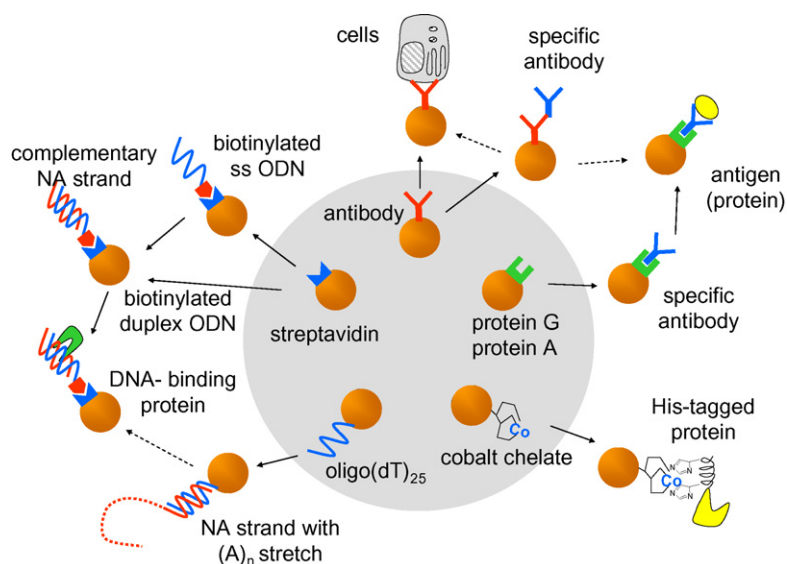


Fig. 1. Magnetic beads (MBs) as versatile tools for bioassays. Beads modified with various recognition elements (examples of commercially available MBs shown on the gray background) can be used for specific bioaffinity capture of different molecules. For example, beads bearing covalently attached oligo(dT)₂₅ chains can bind nucleic acid involving (A)_n stretches, including natural eukaryotic mRNAs or tDNAs tagged with (A)_n adaptors. Streptavidin-coated beads are suitable for capturing any biotinylated molecules, including ss or ds nucleic acids, aptamers, peptides, proteins, etc. Antibodies can be attached to the beads either via a direct covalent linkage, or via specific antibody binding proteins such as protein A or protein G (beads functionalized with the latter proteins can be further modified with various antibodies on demand). Other affinity ligands attached to MBs are suitable for specific capture of tagged recombinant proteins (such as metal-affinity cobalt chelate for histidine tags). Biomolecules captured at the MB surface can serve as a biorecognition layer for interaction with other molecules. For example, a biotinylated ssODN immobilized at streptavidin-coated beads features a hybridization probe for complementary sequence, the dsDNA can represent a target for a DNA-binding protein, antibody can bind a specific antigen (including antigens exposed at surfaces of whole cells), immobilized protein (e.g., via antibody, his-tag or biotin–streptavidin linkage) can interact with a specific nucleic acid or another protein, etc.

well as specific biomolecules, such as nucleic acids (NAs) and proteins [4–11] (Fig. 1). Magnetic methods rely on efficient separation of micrometer-sized paramagnetic or ferromagnetic particles from biological or chemical media. Superparamagnetic particles become magnetic under a strong magnetic field but retain no residual magnetism in the absence of magnetic field. These particles do not interact with each other when removed from the magnetic field. The diameter of the particles is usually between 0.5 and 10 μm . Monodisperse spherical particles (beads) with hydrophilic surfaces are particularly convenient for NAs, providing reproducible magnetic separation. Magnetic beads (MBs) can be prepared in various ways. Usually particles susceptible to magnetism, such as iron oxide, are coated with biological or synthetic polymers [4].

A number of companies offer MBs and kits optimally adjusted for the desired application. The matrices of the magnetic particles are usually composed of agarose, cellulose, silica, silane, porous glass, mica, or polystyrene. Magnetic separators enabling automated handling of the beads are commercially available [4]. With the use of such systems a rapid diagnosis of viral infections [9], separation of specific RNAs [5,11], and extraction of DNA and RNA from various media [7,8,10] is possible. New magnetic particles with improved properties, suitable for diagnostics in microbiology [12], cell isolation [13] and other purposes, were developed. Recently a list of 12 companies and their products was published [4], and it can be expected that materials with more specific binding properties and better separation will soon be developed. Both scale-up procedures for the purification of large volumes of biomacromolecules and miniaturization for sensors and lab-on-a-chip techniques can be foreseen [4,14].

This article will focus on the use of magnetic particles in the sensing of NAs and proteins with electrochemical detection. Special attention will be paid to the sensing of DNA hybridization and DNA–protein interactions.

2. Electrochemistry of nucleic acids

2.1. Intrinsic electroactivity of DNA and RNA

2.1.1. History

Research into the electrochemistry of NAs began almost 50 years ago [15,16] with oscillographic polarography at controlled ac (constant ac chronopotentiometry, according to the present nomenclature). This method proved suitable to indicate reduction and oxidation of bases [16,17] and their ability to form sparingly soluble compounds with the electrode mercury [18–20], but it was also sensitive to changes in DNA structure [17,21]. Soon other polarographic methods, such as pulse- and ac polarography, followed by cyclic and square wave voltammetries, were applied [22–24]. Pulse polarography was particularly useful in the studies of changes in DNA structure, including DNA premelting and DNA damage [25,26]. For about 2 decades only mercury electrodes were used in the analysis of NAs. It was Brabec from our laboratory who then introduced carbon electrodes to study the electrooxidation of NAs [27]. In the recent

decade the electrochemistry of NAs was the subject of numerous reviews (for example Refs. [24,28–38]) dealing mainly with electrochemical DNA hybridization sensors. Here we wish to briefly summarize the principles of NA electrochemistry, including intrinsic electroactivity and labeling of NAs as well as the principles of DNA hybridization sensors, and then to focus on the recent development in the use of MBs in electrochemical NA and protein sensors.

2.1.2. Electrodes

Various electrodes, such as gold, platinum, copper, silver, and indium tin oxide electrodes, were applied to analyze DNAs labeled with electroactive markers or mediated oxidation of guanine (G) residues (reviewed in Ref. [30]), but they were little used to investigate the intrinsic electroactivity of NAs. The potential window in which most solid electrodes can be used is shifted by about 1 V to more positive values as compared with mercury electrodes (operating between 0 and -2 V against the SCE). Solid electrodes are thus better suited for studying NA oxidation, while Hg electrodes are better for investigating NA reduction. Moreover, the atomically smooth and highly reproducible surface of liquid mercury is very well suited for densely packed self-assembled monolayers (SAM) of thiolated DNAs [39] as well as for ac impedance measurements, providing information about DNA adsorption/desorption properties [40].

2.1.3. Sources of intrinsic electroactivity of nucleic acids

Among the nucleotide residues, only the bases undergo reduction at Hg electrodes and oxidation at carbon electrodes, while the phosphate and sugar residues are inactive at these electrodes [30]. Sugar residues in nucleotides are subjected to oxidation at copper electrodes by sinusoidal voltammetry [41]. All NA's bases, many other purines and pyrimidines, adenine (A) and G nucleosides and nucleotides as well as pseudouridine form sparingly soluble compounds with mercury and can be determined by cathodic stripping voltammetry at nanomolar concentrations [18,42–44]. The sensitivity of the determination of the bases can be greatly increased in the presence of copper [45–49]. A and G residues in NAs are oxidized at carbon electrodes; however, their voltammetric peaks were poorly developed, providing insufficient sensitivity in DNA analysis. In the spring of 1995, EP visited for the first time the laboratory of Professor J. Wang (JW) at the New Mexico State University in Las Cruces, NM, to give a seminar. His visit developed, however, in a different way. In that time JW became interested in NAs and wanted to apply constant current chronopotentiometric stripping analysis (CPSA) in DNA research. Soon it became clear that collaboration of JW and EP might be of mutual benefit. The experiments for a first collaborative paper on CPSA of RNA were immediately started and writing of the paper was finished by exchanging faxes during EP's travel through the United States [50]. CPSA with a sophisticated baseline correction proved to be much more convenient than linear sweep (LSV) or differential pulse voltammetry (DPV) for the analysis of DNA and RNA at carbon electrodes. Using CPSA, well-developed oxidation peaks of DNA and RNA at low concentrations were obtained (Fig. 2). This method as well as the square wave voltammetry (SWV)

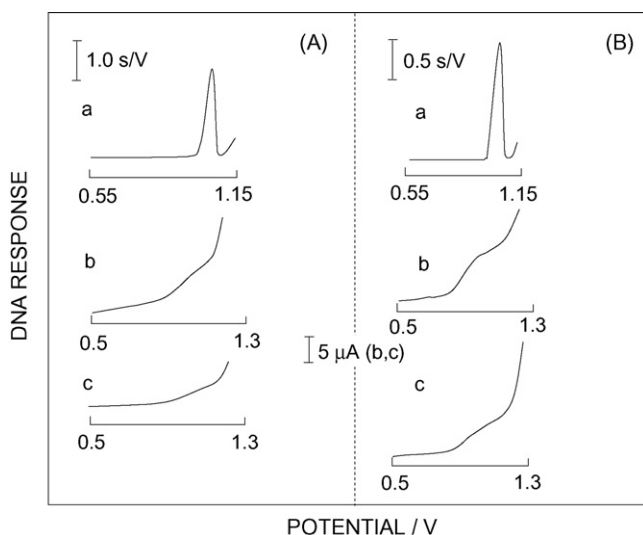


Fig. 2. Constant current chronopotentiograms (a), differential pulse (b), and linear scan (c) voltammograms for $15 \mu\text{g mL}^{-1}$ supercoiled plasmid DNA (A) and $15 \mu\text{g mL}^{-1}$ linearized DNA (B) at carbon paste electrodes. The anodic signal corresponds to electrochemical oxidation of DNA G residues. Adapted with permission from Ref. [52]; copyright 1996 Elsevier.

is at present the methods of choice to study the oxidation of A and G residues in NAs. Oxidation of pyrimidine bases at highly positive potentials was recently reported by Brett [51]. Between 1995 and 1998 EP visited JW's laboratory once or twice a year and his visits resulted in a number of papers based mostly on CPSA [33,50,52–64]. The application of CPSA for the oxidation of peptides and proteins at carbon electrodes appears to be particularly interesting. Similarly to the results obtained with DNA and RNA, this method gave better sensitivity and better resolution of the oxidation signals of tyrosine and tryptophan residues in peptides or proteins than LSV or DPV [53].

At Hg electrodes cytosine (C) and A residues are reduced in single-stranded (ss) DNA and RNA close to -1.4 V at neutral pH. Reduction of thymine (T) and uracil was reported only in nonaqueous media at highly negative potentials. In CV and other cyclic modes, G yields an anodic peak close to -0.3 V , for which oxidation of the G reduction product formed at the highly negative potentials of the background discharge is responsible (Fig. 3).

2.1.4. Effects of nucleic acid structure

Bases are located in the interior of the DNA (or RNA) duplex structures forming a part of the Watson–Crick hydrogen bonding (Fig. 3). The absence of accessible bases at the surface of double-helical NA molecules greatly influences electrochemical responses of these molecules. Native calf thymus DNA showed no dc polarographic wave at neutral pH, in contrast to denatured ssDNA which yielded a wave at about -1.4 V under the same conditions [65]. Also, the non-faradaic capacity signals of DNA were highly sensitive to changes in DNA structure. The strong influence of DNA structure on electrochemical signals obtained by various voltammetric methods and by constant current chronopotentiometry [66] makes the mercury electrodes convenient for studies of DNA structural

transitions and detection of local conformational changes. These electrodes sensitively reflect single strand interruptions in linear and circular DNA molecules [26], differences in the superhelix density, and superhelix density-dependent structural transitions in supercoiled DNAs [24,67].

At a wide range of potentials, the double-helical structure of adsorbed DNA is not substantially affected. In contrast, in a narrow potential range around -1.2 V , relatively slow unwinding of double-stranded (ds) DNA was observed in long linear DNA molecules [24,68]. No unwinding was detected in covalently closed circular DNAs (which cannot unwind for topological reasons) under the same conditions. Mercury electrodes are able to discriminate between DNA and RNA; cathodic voltammetric peaks were used for sensitive determination of traces of RNA in a large excess of DNA [69,70]. The anodic peak of G at mercury electrodes and the oxidation peaks of A and G at carbon electrodes are less sensitive to changes in DNA structure. There were recently some reports about G oxidation at gold electrodes [71] but no systematic studies regarding the effect of DNA structure or application in DNA sensors were published. When working with gold and some other solid electrodes and in DNA sensors nucleic acids are usually labeled.

2.2. Labeling of DNA with electroactive markers

The oxidation and reduction signals of unlabeled NAs are electrochemically irreversible, occurring at highly positive or highly negative potentials. Electroactive labels were introduced into DNA to increase the sensitivity of the analysis and/or to obtain signals at potentials closer to the potential of zero charge. Electroactive osmium markers were introduced into DNA already at the beginning of the 1980s [72–75]. To our knowledge they were the first electroactive species covalently bound to DNA.

2.2.1. Osmium tetroxide complexes

Osmium tetroxide complexes with nitrogen ligands [Os(VIII),L] bind to pyrimidine bases in ssDNA and RNA through the addition to the 5,6 double bond of the pyrimidine ring (Fig. 4A). In this reaction T reacts much faster than C. DNA modification with Os(VIII),L is fast and simple and can be performed under conditions close to physiological [72–77] in an average biochemical or biological laboratory without any special equipment. Os(VIII),L-modified DNA produces a signal at about -1.2 V at liquid mercury and Hg meniscus-modified solid amalgam electrodes [78]. This signal is due to the catalytic hydrogen evolution, detecting ssDNA at concentrations $<1 \text{ ng mL}^{-1}$. In addition, three redox couples between about 0 and -0.6 V are produced at mercury and carbon electrodes [74,77,79–83]. The reaction mixture of Os(VIII),L with DNA can be placed directly on carbon electrodes to detect the DNA–Os,L adduct [80,82].

The Os(VIII),L proved suitable for the end-labeling of signaling (reporter) probes (RPs) composed of a recognition segment (complementary to the target sequence) and an oligo(T) tail (to be modified with the osmium marker) [83]. Longer oligo(T)

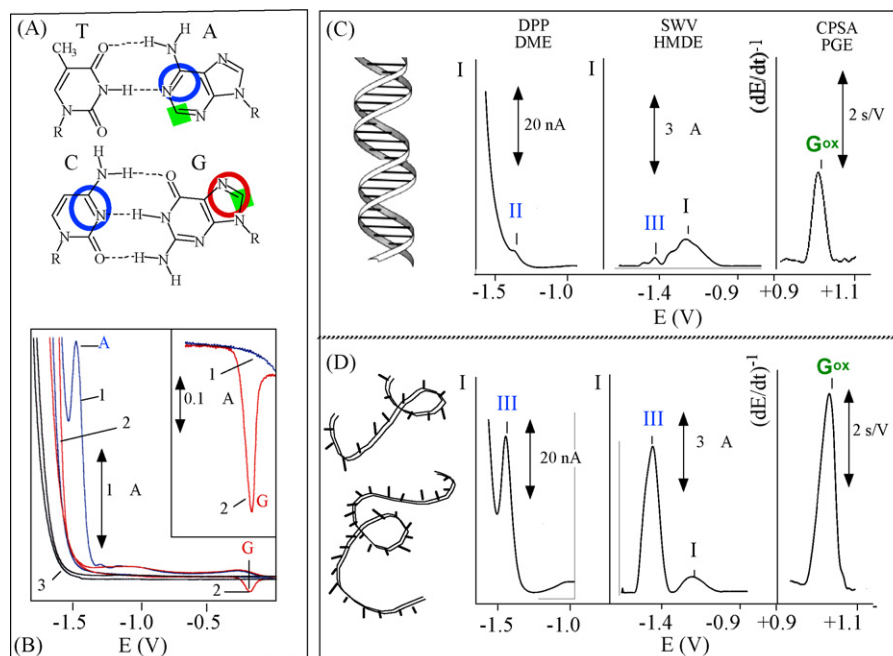


Fig. 3. Reduction and oxidation of ss and ds nucleic acids at electrodes. (A) Schematic representation of Watson-Crick base pairs and electroactive groups. Primary reduction and oxidation sites at mercury (circles) and carbon electrodes (rectangles) are shown. (B) Cyclic voltammograms of biosynthetic polyribonucleotides ($100 \mu\text{g mL}^{-1}$) measured at the hanging mercury drop electrode (HMDE). (1) Polyadenylic acid; (2) polyguanylic acid; (3) background electrolyte; inset, detail of peak G. (C and D) Redox signals: (C) obtained with $100 \mu\text{g mL}^{-1}$ of native (ds) or (D) with $50 \mu\text{g mL}^{-1}$ of denatured ssDNA by differential pulse polarography at the static dropping mercury electrode, by adsorptive stripping SWV at an HMDE, and by CPSA at a pyrolytic graphite electrode. Adapted with permission from Ref. [103]. Copyright 2001 American Chemical Society.

tails can accommodate multiple osmium adducts, resulting in a higher sensitivity of the probe detection. Since purine bases do not react with the Os(VIII),L , the end-labeling of probes with homopurinic recognition segments is easy. When the recognition stretch contains all four bases, it has to be protected during the modification reaction by a complementary strand [84,85]. Redox potentials of the osmium markers can be modulated via choice of the nitrogenous ligand which has been utilized in the technique of “multicolor” DNA labeling [84] (Fig. 4).

2.2.2. Osmium(VI) complexes

In contrast to Os(VIII),L , potassium osmate, $\text{K}_2\text{Os(VI)O}_2(\text{OH})_4$, in the presence of nitrogen ligands does not react with NA's bases. Recently we have shown that sugar residues can be modified by Os(VI) , 2,2'-bipyridine [Os(VI),bipy]. Sugar- Os(VI),bipy -modified ribosides are electroactive, producing similar but not identical electrochemical responses as the base-modified ribosides and DNAs [86,87]. These results thus give a promise for a facile labeling of oligoribonucleotides and natural RNAs at their unphosphorylated 3'-ends. Our preliminary results suggest that such labeling need not be limited to Os(VI),bipy , but that also other nitrogen ligands can be employed.

2.2.3. Ferrocene and other labels

Ferrocene has been perhaps the most frequently used electroactive label in DNA hybridization sensors [88–98]. Ferrocene-labeled synthetic oligodeoxynucleotides (ODNs)

were prepared by covalent linkage of a ferrocenyl group to the amino hexyl-terminated ODN [92,99]. Uracine-conjugated ferrocene ODNs were synthesized and ferrocene-labeled signaling (reporter) probes (RPs) with different redox potentials were prepared for reliable detection of point mutations in DNA [96,97]. Other compounds, such as daunomycin [100], viologen and thionine [101], were used as electroactive labels of ODNs. Ferrocene and other labels mentioned above were usually introduced in oligonucleotides by means of solid-state organic chemistry. Using this approach it may be difficult to label longer NAs, such as plasmid and chromosomal DNAs, viral RNAs, etc. The introduction of electroactive labels into DNA by means of enzymes, such as DNA polymerases and pre-labeled nucleotide triphosphates [102], is a new promising technique. End-labeling of DNA with thiols, biotin and other groups was also used (see below).

3. Electrochemical sensors for DNA hybridization

DNA hybridization is based on the ability of ssDNA to recognize its counterpart strand with a complementary nucleotide sequence. In DNA hybridization sensors ssDNA with a defined nucleotide sequence (probe DNA) is immobilized on a surface. The probe is challenged with another ssDNA in solution, whose sequence is tested (target DNA, tDNA). If the sequence is complementary to the DNA probe, hybrid dsDNA is formed.

For almost one decade both DNA hybridization and electrochemical detection were performed at solid electrodes (mostly gold or carbon electrodes). The hybridization event, i.e. for-

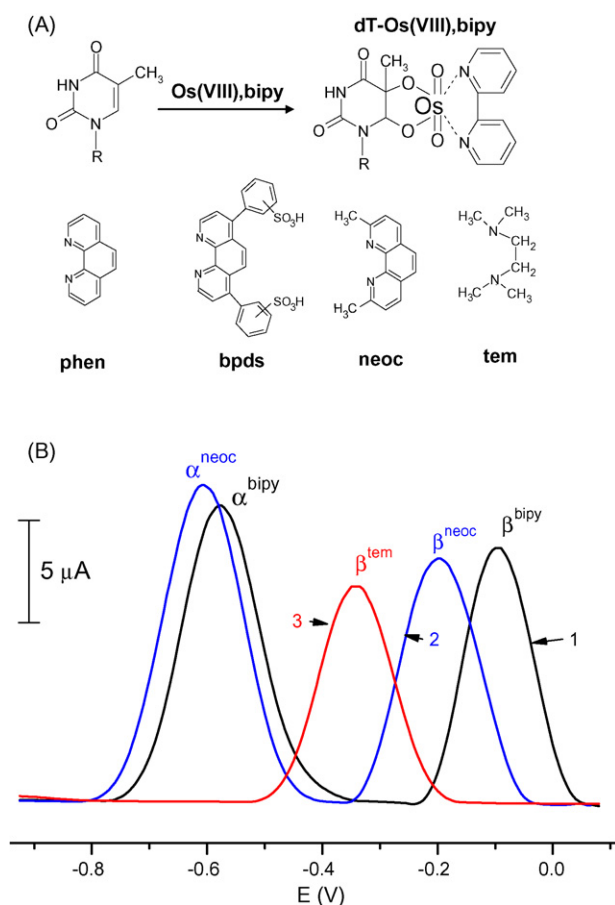


Fig. 4. (A) Formation of the covalent adduct of a DNA thymine residue and the osmium tetroxide, 2,2'-bipyridine [Os(VIII),bipy] complex; ligands that can replace bipy in the complex/adduct: 1,10-phenanthroline (phen); bathophenanthroline disulfonic acid (bpds); 2,9-dimethyl 1,10-phenanthroline (neocuproine, neoc); *N,N,N',N'*-tetramethylethylenediamine (tem). (B) Sections of baseline-subtracted square-wave voltammograms of a 380 nM synthetic oligonucleotide (ODN) modified with various osmium tetroxide complexes: (1) Os(VIII),bipy; (2) Os(VIII),neoc; (3) Os(VIII),tem. Adapted with permission from Ref. [84]; copyright 2007 American Chemical Society.

mation of the DNA duplex (from ss probe and ss tDNAs), was detected in various ways, including the use of redox indicators binding preferentially to dsDNA, labeling of tDNA or RPs with electroactive markers or enzymes, etc. [29,30,103]. This arrangement was simple and efficient when working with model synthetic ODNs, but it had some disadvantages, particularly when long probes and long tDNAs were used. For example, redox indicators, such as intercalators and groove binders (binding preferentially to dsDNA but also in a lesser extent to ssDNA), produced high background signals. Immobilization of probe DNA at electrode surfaces, blocking and interfacing the electrode (transducer) surface, and other details were already reviewed [35,103].

3.1. Detection of DNA point mutations and peptide nucleic acid

The relation between some diseases and single base mutations (point mutations) is becoming more and more evident.

The detection of a small change in DNA due to the presence of a single base mismatch in the duplex is not an easy task requiring specific approaches and methods. In 1995, the development of electrochemical DNA hybridization sensors was at the beginning and no method of detection of DNA point mutations by these sensors was available. Again, an interesting coincidence helped to solve the problem. In 1995, EP lectured at the EMBO course on DNA probing by metal complexes. This course was organized in Copenhagen by D.M.J. Lilley and P. Nielsen, the inventor of peptide nucleic acid (PNA) [104]. At the same time JW was lecturing at another place in Copenhagen. JW and EP met in the city and discussed the electrochemistry of NA, PNA, and their further collaboration. PNA was considered as one of the candidates for diagnostic and therapeutic application in medicine. In PNA, the entire sugar–phosphate backbone is replaced by *N*-(2-amino-ethyl) glycine units. PNA is achiral and has an electrically neutral backbone. It has a higher binding affinity to complementary DNA and RNA than unmodified ODNs. After his return from Denmark, JW soon expressed his interest in PNA. JW, EP, and P. Nielsen joined their efforts in utilizing PNA for the electrochemical detection of DNA point mutation. Early in 1996, a simple method using PNA as a recognition layer, based on CPSA, carbon electrodes and a redox indicator, was submitted to *J. Am. Chem. Soc.* [61]. This paper marked the first electrochemical detection of point mutation and the first application of PNA as a recognition layer in an electrochemical DNA hybridization sensor. Later we published, together with JW, papers on the detection of point mutation in the tumor suppressor p53 gene [105] and on PNA oxidation and adsorption at carbon electrodes [61], reduction [106], and adsorption/desorption at mercury electrodes [56,105]. Very recently a PNA-based DNA hybridization sensor was proposed by Steichen et al. [107]. In this sensor, PNA end-labeled with cysteine is immobilized on a gold electrode. PNA alone does not bind the $[\text{Ru}(\text{NH}_3)_3]^{3+}$ cation but when a PNA·DNA hybrid is formed, its presence is indicated by the binding of $[\text{Ru}(\text{NH}_3)_3]^{3+}$ to negatively charged tDNA. A single base mismatch A2143G in the 23S rRNA gene of *Helicobacter pylori* was easily detected. Changes in the DNA structure immobilized at the electrode surface have also been utilized in DNA sensors. Particularly the surface-attached molecular beacons attracted great attention (Refs. [88,89,108]; reviewed in Refs. [30,109]).

4. Magnetic beads in electrochemical nucleic acids and protein sensing

4.1. Double-surface technique

The process of DNA hybridization, which is based on recognition of the complementary DNA single strands, greatly differs from the electrochemical detection of the hybridization event. It is therefore not surprising that the properties of the optimum hybridization (H) surface can hardly be the same as those of the optimum surface for electrochemical DNA detection. For example, (a) nonspecific DNA adsorption during DNA hybridization should be minimized. On the other hand, adsorption of DNA at the detection electrode (DE) is convenient because accumu-

lation of DNA at the DE can result in increased sensitivity of DNA detection; (b) the DE should be small (to be able to detect a small number of DNA molecules which underwent DNA hybridization). In contrast, the surface H should be relatively large (to accommodate a large number of the probe DNA molecules for DNA hybridization); (c) for the DNA hybridization the DE should be blocked or interfaced to increase the selectivity of DNA hybridization and to eliminate false positive signals. On the other hand, the blocking or interfacing of DE may decrease the sensitivity of DNA determination, etc. Optimization of hybridization and detection at a single DE surface is thus not an easy task. At the beginning of 2000, these problems prompted the development of a new approach in our laboratory, in which DNA hybridization was performed at one surface (surface H) and electrochemical detection on another surface, DE. As the surface H we used commercially available MBs (Fig. 1). We were quite excited with the results of this new approach and we believed that we were the only ones who got such a nice idea. At that time we collaborated with a company which was interested in patenting the new approach. This prevented us from a fast publication of our results. In November 2000 (at the time of presidential elections), EP visited JW's laboratory and to his surprise a magnetic separator arrived to the laboratory during his visit indicating that Joe got the same or a similar idea, but we still believed that in Brno we were ahead of Las Cruces. This, of course, was our mistake. In the summer of 2000, EP was invited to act as Guest Editor of a special issue of *Talanta* on "Electrochemistry of nucleic acids and development of electrochemical DNA sensors" and we planned to publish our paper on the double-surface technique (DST) in this issue. In June 2001, at the XVIth Bioelectrochemistry Symposium in Bratislava, both Joe Wang and we independently reported for the first time on the new DST for electrochemical DNA hybridization. Since that time we have been following the rule: "Joe is faster than we are". In fact he is much faster!

4.2. Sensing DNA hybridization

In the following years DST has been doing very well. Due to minimization of nonspecific DNA adsorption at the MBs, a very high specificity of DNA hybridization was achieved. The highly efficient separation of specific NA strands (tDNA and/or RPs, Fig. 5) can easily be combined with label-free [47–49,110,111] as well as label-based electrochemical NA detection. Covalent DNA labeling with osmium tetroxide complexes (see Section 2.2.1) proved convenient in various applications [78,80,83,84]. More recently, other types of DNA labels based on transition metal complexes covalently tethered to nucleobases have been developed [112] and successfully applied in the DSTs (M. Fojta, M. Hocek et al., unpublished). The enzymes tethered to tDNA and/or to RPs (mostly via streptavidin–biotin linkage, Fig. 5C) represent another type of powerful DNA markers [113–117]. Moreover, JW mastered the DST by combining it with metal and semiconductor nanoparticles, nanowires, electroactive beads, carbon nanotubes, etc., reaching the highest sensitivity of DNA detection in electrochemical sensors [114,118–123]. As a major part of his work was recently reviewed [31,124], here only some

examples will be presented and various approaches showing the advantages of DST will be given.

4.2.1. Choice of the detection electrode and electrochemical technique

In DST it is advantageous that an optimum DE and electroanalytical technique can be chosen only with respect to the given electrode process. Thus high sensitivity of stripping techniques, based on DNA (and/or the DNA marker) accumulation at the DE, can be fully utilized. In a variety of protocols the hybridized (accumulated and separated) species have been detached from the MB, followed by their stripping detection (Fig. 5). It was shown that mercury electrodes with flatly adsorbed probe DNA or RNA are not convenient for hybridization due to a strong adsorption of the bases to the hydrophobic mercury surface [55]. In DST this obstacle does not prevent the use of the mercury, mercury-film and solid amalgam electrodes, which are convenient for anodic stripping of metals and cathodic stripping of NA bases forming sparingly soluble complexes with mercury [110,125] or with copper(I) [49,126] ions. Purine bases were released from DNA by acid treatment and determined at sub-nanomolar concentrations at silver [110,125,126], copper [49], platinum, or gold [48] amalgam electrodes. The Cu(I)-purine complexes were also efficiently accumulated at carbon electrodes, resulting in a considerable enhancement of the G and A anodic signals (which are well-resolved, thus enabling relative quantification of purine bases in an NA sample) [47,127].

Nanoparticle tags in combination with the stripping determination of the nanoparticle material (sulfides of cadmium or other metals) dissolved in a suitable solvent were applied in DNA hybridization sensing [128,129]. The nanoparticle-based systems have been successfully combined with signal amplification via nanoparticle-promoted accumulation of the tracer molecules (atoms). For example, precipitation of silver on gold [128] or cadmium on CdS [129] nanoparticle tags tethered to DNA was used to enlarge the nanoparticle tag and amplify the DNA signal. A considerable signal amplification can be achieved via employment of biocatalysis (utilization of enzyme tags, see Section 4.2.6) [113–115].

In the MB-based assays, the captured (hybridized) NA strands need not necessarily be released from the beads prior to analysis. When using enzyme labels, procedures similar to the well-known ELISA technique can be applied, involving transfer of the MBs with the entire molecular "sandwich" into the substrate solution, followed by enzymatic production of an electrochemically active indicator (Fig. 5C). In addition, both enzyme tags [130] and nanoparticle tracers [131,132] can be detected after magnetic attraction of the beads to the DE surface. In this respect, a combination of the MBs with "magnetocomposite electrodes" [111,132,133] represents an elegant concept of magnetically switchable devices [134–136]. For example, in "magnetic collection" of the magnetic-bead/DNA-hybrid/CdS-tracer assembly (onto a thick-film carbon electrode), the amplification features of nanoparticle/DNA assemblies and highly sensitive potentiometric stripping detection of cadmium were combined [131].

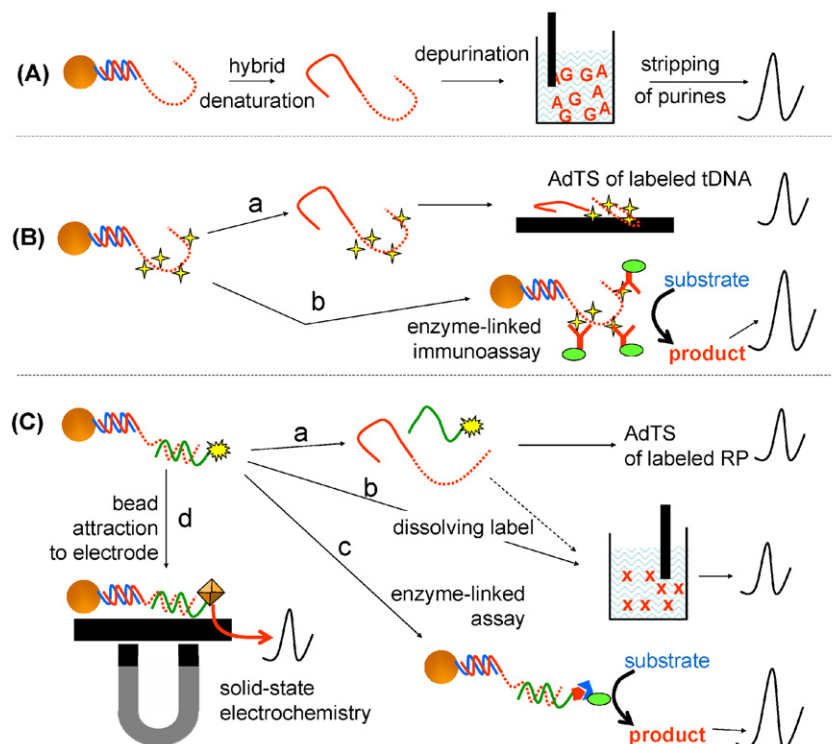


Fig. 5. Some detection principles used in the MB-based electrochemical double-surface DNA hybridization techniques. (A) Label-free detection of target DNA (tDNA). After hybridization with a capture probe at the beads and magnetoseparation, the DNA is detached from the MBs, treated with perchloric acid to release purine bases, and the latter are determined using highly sensitive stripping voltammetric techniques. (B) Labeling of tDNA. Labels such as osmium tetroxide complexes (Os,L) are covalently attached to the tDNA strand outside the segment recognized by the capture probe. After hybridization and separation, the electroactive tags are determined electrochemically (e.g., by ex situ adsorptive stripping voltammetry (a)). Alternatively, electrochemical enzyme-linked immunoassay can be used for detection of labeled tDNA at the MB surface (b). (C) "Sandwich" hybridization assays using labeled reporter probes (RP). Target DNA is attached to MBs via the capture probe, followed by hybridization with RP. Detection of RP tags can be achieved after detachment from the MBs using the ex situ stripping measurements (used e.g., for Os,L labeled RPs (a)). Nanoparticle-based DNA tracers or "electroactive beads" can be determined after dissolution in a suitable medium (b). For detection of biotin tags, enzyme-linked assays employing streptavidin-enzyme conjugates are particularly convenient (c). Metal nanoparticles as well as metal sulfide nanocrystals can be detected via solid-state electrochemical measurements after magnetic attraction of the entire MB-tDNA-RP assembly to an electrode. (Approaches mentioned as examples of RP-based techniques can in principle be applied for labeling/detection of tDNA and vice versa.)

4.2.2. Nanoparticles and nanocrystals

Semiconductor (*quantum dots*) nanocrystals generated considerable interest in optical DNA detection [137] due to their unique (tunable-electronic) properties. Recently, such inorganic

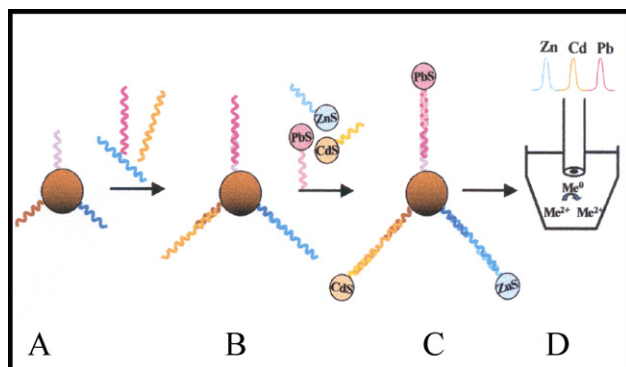


Fig. 6. A multitarget electrical DNA detection protocol based on different inorganic colloid nanocrystal ("quantum dots", QD) tracers. (A) Introduction of probe-modified MBs. (B) Hybridization with DNA targets. (C) Second hybridization with QD-labeled probes. (D) Dissolution of QDs and electrochemical detection. Adapted with permission from Ref. [139]; copyright 2003 American Chemical Society.

crystals were utilized for enhanced electrical DNA detection [123,129,138]. A *photoelectrochemical* transduction of DNA sensing events in connection with DNA-cross-linked CdS nanoparticle arrays was reported [138]. The electrostatic binding of the $[\text{Ru}(\text{NH}_3)_6]^{3+}$ to the dsDNA provided a tunneling route for the electron-band electrons and increased photocurrents. Wang et al. reported detection of DNA hybridization using CdS nanoparticle tags and stripping Cd determination [129]. A nanoparticle-promoted Cd precipitation was used to amplify the stripping DNA hybridization signal.

The application of other inorganic nanocrystals such as PbS and ZnS paved the way for a new *coding* technology affording simultaneous detection of multiple DNA nucleotide sequences. Nanocrystal tags with diverse redox potentials [139] displayed well-resolved stripping responses of ZnS, CdS, and PbS at the mercury-coated glassy carbon electrode. Functionalizing these nanocrystal tags with thiolated ODN (HS-ODN) probes offered distinct electrical hybridization signals for the corresponding DNA sequences (Fig. 6). The application of further nanocrystals, such as InAs and GaAs, can provide multitarget detection and a high-throughput massively parallel assay if microtiter plates or multichannel microchips are used [31].

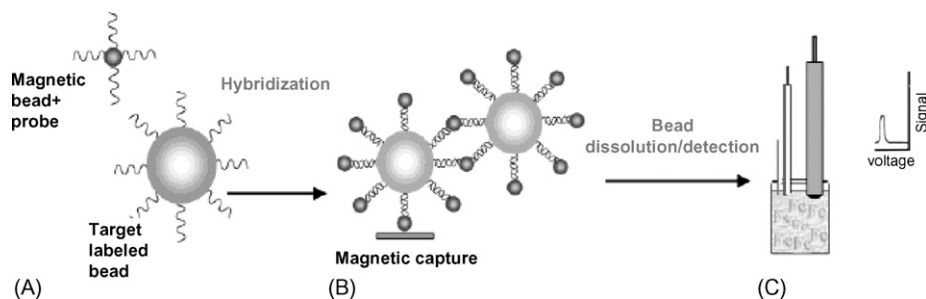


Fig. 7. Utilization of “electroactive beads” (polystyrene spheres loaded with the electroactive marker ferrocene) in a magnetic DNA hybridization assay: (A) Introduction of probe-coated MBs and target-labeled electroactive beads. (B) Hybridization and magnetic separation. (C) Dissolution of the spheres in acetonitrile, release of the marker, and its CPSCA detection at a glassy carbon electrode. Adapted with permission from Ref. [142]; copyright 2003 American Chemical Society.

In Wang’s laboratory large particle-based libraries for electrical coding were developed, based on judiciously designed encoded “identification beads” [140] or striped metal rods [141]. Such beads or rods can provide a large number of stripping signatures for a reliable identification of a large number of specific DNA nucleotide sequences. Capping the rod with gold may facilitate functionalization of the rod with an HS-ODN probe. Large numbers of usable codes could be generated using 4–5 different peak potentials and 3–4 different current intensities. Such “identification beads” show great promise for authenticity testing and identification of counterfeit products.

Indium is not normally present in biological samples or reagents and offers an attractive electrochemical stripping behavior. Using indium for plating resulted in a low detection limit (250 zmol). Derivative-chronopotentiometric tracing of the indium tracer was realized through a “magnetic” collection of the DNA-linked particle assembly onto a thick-film carbon electrode.

4.2.3. Ultrasensitive assays using particle-based multiple amplification avenues

Some amplification processes such as various kinds of catalysis were already reviewed [31,134]. J. Wang invented *polymeric microbeads* carrying multiple redox tracers internally [142] (Fig. 7) or externally [143]. Such microbeads greatly amplify the electrochemical signal, meeting the high sensitivity demands of DNA biosensors. Polystyrene microbeads coated with streptavidin were used for the binding of about 80 metal nanoparticles per bead. A combination of these polymeric microbeads with the stripping detection and catalytic enlargement of the multiple gold-particle tags resulted in a dramatic enhancement of sensitivity [143]. An ultrasensitive DNA hybridization detection utilizing polystyrene microspheres containing $[\text{Ru}(\text{bipy})_3]^{2+}$ combined with electrogenerated chemiluminescence was proposed by Miao and Bard [144]. Combined multiple amplification pathways, based on enzyme-functionalized liposomes and accumulation of the biocatalytic-reaction product, for ultrasensitive DNA CPS assays was developed in Willner’s laboratory [145]. Gold nanoparticles used as carriers of 6-ferrocenylhexaethiol in combination with a sandwich hybridization assay represented another method of amplification of the electrochemical signal [146]. The ferrocene/Au-nanoparticle conjugates were positioned directly in proximity to the electrode allowing

facile electron transfer. A detection limit of 10 amol was obtained.

4.2.4. Carbon nanotubes

Carbon nanotubes (CNTs) have recently attracted considerable attention due to their unique structural, electronic, mechanical, and chemical properties [147,148]. CNT-modified carbon electrodes were applied to improve electrochemical detection of purines and NAs [149,150]. Vertically aligned multiwalled CNTs embedded in SiO_2 were used for the construction of electrode arrays applicable for DNA detection [151]. Apart from utilization of CNTs as electrode surface modifiers, CNT-based tags tethered to ODN probes were used in detecting DNA hybridization [114,123]. These applications have taken the advantage of the CNTs, which can accommodate many electroactive or enzyme tracer entities per nanotube. Using a 96-well microplate platform, Wang et al. demonstrated [123] a considerable signal enhancement due to the loading of about 500 CdS nanoparticles per CNT bound to an ODN probe. In another paper by the JW group [114], ODN probes coupled to CNTs loaded with multiple alkaline phosphatase molecules were applied in connection with the MB-based DST (Fig. 8). This approach resulted in an ultrasensitive DNA detection allowing the determination of less than 1000 copies of a complementary nucleotide sequence.

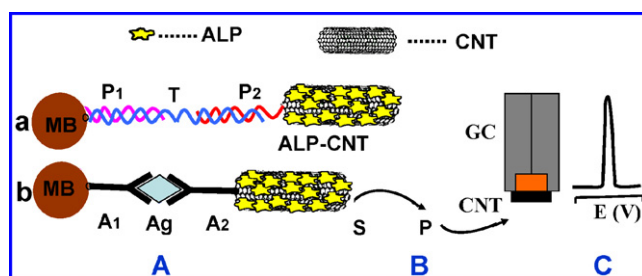


Fig. 8. Ultrasensitive biosensing of DNA or proteins using enzyme-loaded carbon nanotubes (CNT). (A) Capture of the ALP-loaded CNT tags to the streptavidin-modified MBs by a sandwich DNA hybridization (a) or Ab–Ag–Ab interaction (b). (B) Enzymatic reaction. (C) Electrochemical detection of the product of the enzymatic reaction at the CNT-modified glassy carbon electrode; MB, magnetic beads; P, DNA probe 1; T, DNA target; P2, DNA probe 2; Ab1, first antibody; Ag, antigen; Ab2, secondary antibody; S and P, substrate and product, respectively, of the enzymatic reaction; GC, glassy carbon electrode; CNT, carbon nanotube layer. Adapted with permission from Ref. [114]; copyright 2004 American Chemical Society.

4.2.5. Enzyme tags

Enzymes proved to be convenient tools in biosensing due to the inherent “biocatalytic” signal amplification: one enzyme molecule can undergo many catalytic turns, thus producing many indicator molecules per hybridization event. For the electrochemical enzyme-linked approaches it is necessary that the electrochemical activity of the substrate differs from that of the product into which it is converted. For example, 1-naphthol yields an oxidation signal at carbon electrodes while 1-naphthyl phosphate does not. If alkaline phosphatase (ALP) is used as the ultimate DNA label (as it was in the early papers on “magnetic” electrochemical DST [113,116]), the latter substance is converted into the former and the signal due to 1-naphthol oxidation appears. ALP belongs to the most frequently employed enzymes in DNA sensing; it has been usually applied as a streptavidin conjugate (to be linked to biotinylated RPs) [115,116,152] or coupled to secondary antibody (in enzyme-linked immunoassay) [113]. Among others, β -galactosidase (releasing phenol from an inactive ester) was used (together with the ALP) by JW et al. [117] to develop a technique for dual DNA coding. Peroxidases have been utilized for DNA labeling and detection using single-surface biosensors [153] or techniques employing polystyrene microtitration wells [154] as well as for antigen labeling in electrochemical MB-based DNA hybridization assays and immunoassay [133,155].

4.2.6. Other

4.2.6.1. Enzyme-linked immunoassays with electrochemical detection. Immunosensors and electrochemical immunoassays represent attractive methods in bioanalysis due to their versatility, high sensitivities, and low detection limits [113,156]. Until 2002 there had been no reports on the electrochemical immunoassay of nucleic acids. This was probably due to low immunological responses of unmodified DNA. Earlier we showed a strong immunogenicity of osmium-modified DNA [157,158]. We utilized the immunogenicity of the Os(VIII),bipy-modified DNA (see Section 2.2.1) and DST to propose an electrochemical DNA hybridization sensor based on an enzyme-linked immunoassay [113]. Paramagnetic Dynabeads Oligo(dT) with a covalently bound (dT)₂₅ probe were used to hybridize with tDNAs containing adenine stretches. The tDNA was modified with Os(VIII),bipy and the immunogenic DNA-Os(VIII),bipy adduct was determined by the enzyme-linked immunoassay using monoclonal antibody OsBP7H8 and a secondary antibody–ALP conjugate. 1-Naphthyl phosphate was used as a substrate and the product (1-naphthol) was measured on carbon electrodes (Fig. 5B). DNA hybridization of long target ODNs (such as 67- and 97-mers) and a DNA PCR product (226 base pairs) were detected at high sensitivity and specificity. All T residues can be modified in tDNA while the complementary target A_n stretch does not react with Os(VIII),bipy (see also Fig. 9). Alternatively an RP can be used with a long T tail in which tens of Ts are modified [83], thus creating a number of sites for the antibody binding.

4.2.6.2. Detection of trinucleotide repeat expansion. Expansions of DNA stretches comprising repeated triplet units are

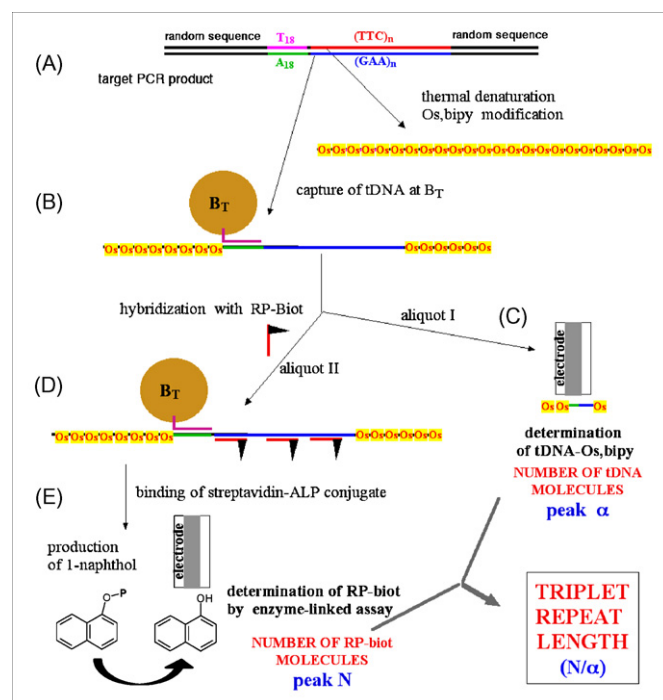


Fig. 9. Detection of (GAA)_n repeat expansion in a PCR-amplified fragment of human frataxin gene. (A) The tDNA is thermally denatured and modified with Os(VIII),bipy. (B) The tDNA strand containing the (GAA)_n sequence is captured at MBs bearing a dT₂₅ capture probe (B_T) via its dA₁₈ stretch. After washing, the B_T suspension is split into two aliquots. (C) Aliquot I: the tDNA-Os(VIII),bipy is released from the B_T and determined using osmium peak α at the carbon electrode. The number of pyrimidine residues modified by Os(VIII),bipy in the GAA-strand of the tDNA is constant and independent of the length of the repeat tract. (D and E) Aliquot II: biotinylated RP (TTC)₁₂ is hybridized with the target (GAA)_n sequence at the beads followed by binding of streptavidin–alkaline phosphatase conjugate. Then the beads are transferred into a solution of 1-naphthyl phosphate which is enzymatically converted to electroactive 1-naphthol yielding anodic peak N at the carbon electrode. The resulting 1-naphthol concentration depends on the number of RP-Biot molecules captured at the B_T, which is directly proportional to the (GAA)_n length. The triplet repeat expansion length is estimated from the peak height ratio N/α . Adapted with permission from Ref. [115]; copyright 2004 American Chemical Society.

related to the pathogenesis of hereditary neurodegenerative diseases such as fragile X syndrome, Huntington’s disease, or Friedreich’s ataxia (FRDA). The molecular diagnostics of these disorders is based on the determination of the number of trinucleotide repeat units in specific genomic loci [115,152,159]. This can be achieved (besides other techniques) via hybridization of the target triplet repeat with a labeled RP spanning several trinucleotide units [115,152]. The electrochemical DSTs proved convenient for this purpose, and particularly for determination of the length of the (GAA)_n triplet repeat related to FRDA [115] (Fig. 9). Biotinylated (TTC)₁₂ RP was hybridized with PCR amplified tDNA immobilized at the MBs via A₁₈ stretch (inherently present in the amplified genomic DNA fragment) forming DNA duplex with a T₂₅ capture probe. A number of hybridized probes were determined via an enzyme-linked assay with streptavidin–ALP conjugate (see Section 4.2.6). To normalize the RP signal, pyrimidine residues within sequences flanking the homopurine (GAA)_n repeat in tDNA were premod-

ified with Os(VIII), bipy and the electroactive labels introduced were determined voltammetrically at carbon electrodes. The length of the triplet expansion was calculated from the ratio of intensities of signals of the hybridized RP/tDNA-Os(VIII), bipy [115] (Fig. 9).

4.2.6.3. “Multicolor” electrochemical coding of nucleotide sequences. Modern “gene chip” analyzers utilize multicolor optical labeling for the recognition and simultaneous analysis of multiple target genes. Highly parallel detection is essential for the development of high-throughput techniques determining the progress in contemporary molecular biology (including application of the new knowledge in biomedicine and other practical areas having impact on human life quality). Electrochemical DNA sensors can offer a parallel analysis of multiple genes as well, provided that individual target sequences yield distinguishable electrochemical signals. This generally requires the application of multiple tags or indicators differing in their redox potentials, each assigned (tethered) to a particular nucleotide sequence (probe). Several “multicolor” (multipotential) DNA coding approaches have recently been introduced in connection with “magnetic” DSTs.

Dual DNA coding with two enzymes, ALP and β -galactosidase, was proposed by Wang et al. [117]. The recognition of two DNA targets was attained through enzymatic production of two electroactive indicators, 1-naphthol and phenol, which can be determined simultaneously via oxidation at carbon electrodes. The same group proposed [139] a technique employing PbS, CdS, and ZnS nanoparticles coupled to different DNA probes (Fig. 6). After hybridization and separation at MBs, the nanoparticles were dissolved in acid and the metals determined sensitively by means of anodic stripping. The presence of particular electrochemical signals indicated the presence of particular nucleotide sequences (featured by synthetic ODNs) complementary to probes for whose labeling the respective nanoparticles were used.

Electrochemically “tunable” metal chelates, covalently attached to DNA or its components, have been developed by several groups. For example, Kuhr and co-workers [160] and Gooding and co-workers [161] employed different ferrocene derivatives as DNA tags in a DNA sequencing technique (using capillary electrophoresis coupled to a sinusoidal-voltammetric detector) [160] or primer extension reactions on SAM-modified gold electrodes [161]. Weizman and Tor introduced [162] “tunable” electroactive DNA tags based on Ru or Os complexes with different, electrically charged or neutral ligands. We have recently proposed [84] another “multicolor” DNA coding technique based on a modification of oligo(T) tails in RPs with osmium tetroxide complexes involving different nitrogenous ligands (Fig. 4). Complexes (DNA adducts) bearing ligands such as 2,2'-bipyridine, 1,10-phenanthroline derivatives or *N,N,N',N'*-tetramethyl ethylenediamine yielded redox signals at carbon electrodes at potentials sufficiently separated to allow differentiation and/or simultaneous detection of several DNA targets via the MB-based double-surface strategy.

4.3. DNA–protein interaction

Considering that NAs and proteins are electroactive at mercury and carbon electrodes, the binding of practically any protein to DNA (or RNA) can be detected by DST. For example, specific DNA can be bound to the MBs and interacted with a protein mixture followed by washing, dissociation of the bound protein, and electrochemical detection. Mercury-containing electrodes give the best sensitivity when the electrocatalytic peak H is used [66,163–165]. Carbon electrodes in combination with CPSA or SWV with efficient baseline correction can be applied for proteins containing oxidizable tyrosine and/or tryptophan residues. Both types of electrodes were used to investigate the interaction of MutS protein with DNA aimed to detect DNA point mutations [164,166]. The MutS protein plays an important role in the DNA repair system in prokaryotic and eukaryotic cells [163] and can be used for the detection of point mutations *in vitro*.

The first electrochemical detection of DNA single base mismatches (point mutations) and insertion/deletions by means of MutS protein was based on DST [164]. Biotinylated DNA duplexes were attached to MBs and interacted with the MutS protein. The DNA-bound protein was released from the beads and determined electrochemically; using peak H and a hanging mercury drop electrode (HMDE), tens of attomoles of this protein were detected [164]. The determination of MutS protein at carbon electrodes was by 2–3 orders of magnitude less sensitive. Recently the analysis of MutS protein at carbon electrodes was optimized [166] and its sensitivity was increased to that obtained earlier using peak H [164]. Adjusting optimum conditions of SWV determination and partial hydrolysis of the protein decreased the detection limit down to 5 pg in 5 μ L of the analyte. In addition to the previously shown single nucleotide polymorphisms, the binding of MutS to AA and CA mismatches was detected electrochemically. These methods [164,165] opened the door to the investigation of NA–protein interactions applicable to a large number of NA-binding proteins.

4.4. Aptamer–protein recognition

In recent years, synthetic nucleic acid-based ligands, *aptamers*, have attracted considerable attention as biorecognition elements binding proteins with high selectivity and affinity [108,130,167–172]. Aptamers may in principle be more convenient than antibodies since they can be completely selected, engineered, and labeled *in vitro*. Besides the utilization of magnetoseparation techniques during the aptamers selection [167], coupling aptamers to magnetic particles has been utilized in the target protein purification [170,171] and detection (using various platforms such as fluorescence [168], surface plasmon resonance [161] or electrochemistry [130,169,172]). Kawde et al. [169] utilized streptavidin-coated MBs with immobilized biotinylated lysozyme-specific aptamer for label-free detection of lysozyme (Fig. 10). The technique based on the oxidation signal of tyrosine and tryptophan in the protein at a pencil graphite electrode allowed determination of the lysozyme in

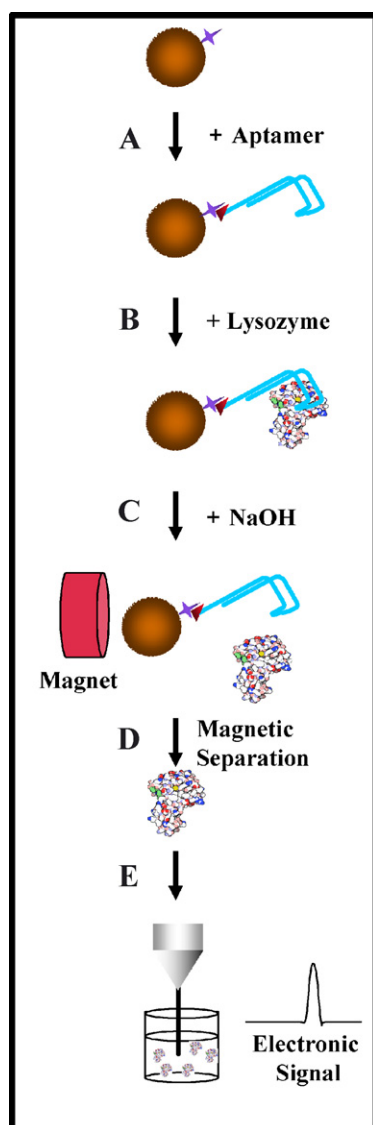


Fig. 10. Detecting aptamer–protein interactions: (a) attachment of the biotinylated anti-lysozyme aptamer to the streptavidin-coated MBs; (b) selective binding of the target protein; (c) alkaline-induced release of the captured protein; (d) magnetic separation; (e) CPSA detection of the released protein in connection to an adsorptive accumulation step. Adapted with permission from Ref. [169]; copyright 2005 Elsevier.

large excesses of other proteins or amino acids. Centi et al. [130] applied an enzyme-linked electrochemical approach for the detection of thrombin via an aptamer sandwich assay. Thrombin was bound to the thrombin aptamer anchored at the MBs, followed by the binding of another aptamer molecule labeled with ALP. The (MB–aptamer)–thrombin–(aptamer–ALP) complex was magnetically attracted to a carbon electrode, followed by enzyme-catalyzed production of 1-naphthol and its electrochemical detection.

4.5. Immunoassays of proteins or other antigens

The “biomagnetic” concept combined with electrochemical detection has been successfully applied in sandwich [114,173] or competitive [155] immunoassays of proteinaceous as well

as small molecule antigens. An ultrasensitive detection of the antigen–antibody interaction was achieved by Wang et al. via application of the ALP-loaded CNTs as labels of the signaling antibody (an analogous technique was used for DNA sensing, see Section 4.2.5, Fig. 8) [114]. An interesting approach exploiting the intrinsic electroactivity of nucleobases at carbon electrodes was introduced by the same group [173]. The latter technique employed polystyrene beads functionalized with synthetic ODNs containing A and/or G nucleobases as electroactive tags tethered to antibodies. After the immunorecognition and separation steps, the ODN tracers were released and acid-hydrolyzed, followed by electrochemical detection of the purine bases at carbon electrodes. Since each polystyrene bead carried many ODN chains, each containing multiple purine bases, a considerable enhancement of the signal responding to the antibody–antigen binding event was achieved.

Alegret and co-workers [155] proposed an electrochemical immunosensing strategy for the detection of pesticides (atrazine). The immunological reaction for the detection of the pesticide was based on a direct competitive assay using a peroxidase (HRP) tracer as the enzymatic label. After the immunochemical reactions, MBs carrying the antibody–antigen complex were attracted to a graphite-epoxy composite magnetosensor, followed by electrochemical detection through a suitable substrate and mediator for the enzyme HRP.

5. Conclusion

There is no doubt that MBs are very useful tools in biochemistry and molecular biology. This article illustrates the usefulness of MBs in electrochemical biosensing and particularly in DNA hybridization sensors. Separating DNA hybridization from the electrochemical detection on DE provides a number of advantages; some of them are mentioned in this paper. It can be expected that others will be shown soon. On the other hand, in order to create a device for practical nucleotide sequencing based on MB, the problem of MB manipulation has to be solved, without making the device too complicated and expensive. Microfluidic systems for MB manipulation are already available but, to our knowledge, they have not yet been combined with a practical DNA hybridization sensor [120,174]. In recent years great progress has been achieved in the development of sensors based on DNA hybridization and detection at single surface sensors. Particularly the sensors utilizing changes in DNA structure and properties after DNA hybridization, such as surface-attached molecular beacons [88,89,108,109], appear attractive. There is no doubt that the race between single- and double-surface techniques is not yet over and surprises and breakthroughs cannot be excluded.

Electrochemical biosensing is certainly not limited to DNA (or RNA) hybridization. NA–protein interactions, protein–protein interactions, and immunoassays are of great importance in biomedicine, proteomics, and in biological sciences in general. The outlooks for the application of MB and electrochemistry in creating new tools for biological research are bright and challenge electrochemists, biochemists, and biologists to join their forces. New powerful methods have almost

always represented a great stimulus for important breakthroughs in science.

Congratulations

We have known Joe Wang as a young hard-working and extremely successful scientist. It is thus surprising for us that he will soon celebrate his 60th birthday. Joe, we wish you, in addition to success, health and happiness, to remain young at least till your 90th birthday!



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References

- [1] J. Marmur, D. Lane, *Proc. Natl. Acad. Sci. U.S.A.* 46 (1960) 453–461.
- [2] J. Marmur, R. Rownd, C.L. Schildkraut, in: J.N. Davidson, W.E. Cohn (Eds.), *Progress in Nucleic Acid Research*, vol. 1, Academic Press, London, 1963, pp. 231–300.
- [3] J. Marmur, *J. Mol. Biol.* 3 (1961) 208–218.
- [4] S. Berensmeier, N. Theilacker, *BioTech* (2007) 6–10.
- [5] A.L. Hei, J.P. Cai, *DNA Cell Biol.* 24 (2005) 479–484.
- [6] D. Horak, B. Rittich, A. Spanova, M.J. Benes, *Polymer* 46 (2005) 1245–1255.
- [7] M.K. Hourfar, U. Michelsen, M. Schmidt, A. Berger, E. Seifried, W.K. Roth, *Clin. Chem.* 51 (2005) 1217–1222.
- [8] M.K. Hourfar, M. Schmidt, E. Seifried, W.K. Roth, *Vox Sanguinis* 89 (2005) 71–76.
- [9] P. Muir, F. Nicholson, M. Jhetam, S. Neogi, J.E. Banatvala, *J. Clin. Microbiol.* 31 (1993) 31–38.
- [10] L. Pichl, A. Heitmann, P. Herzog, J. Oster, H. Smets, V. Schottstedt, *Transfusion* 45 (2005) 1106–1110.
- [11] S.A. Rutjes, R. Italiaander, H. van den Berg, W.J. Lodder, A.M.D. Husman, *Appl. Environ. Microbiol.* 71 (2005) 3734–3740.
- [12] O. Olsvik, T. Popovic, E. Skjerve, K.S. Cudjoe, E. Hornes, J. Ugelstad, M. Uhlen, *Clin. Microbiol. Rev.* 7 (1994) 43–54.
- [13] I. Safarik, M. Safarikova, *J. Chromatogr. B* 722 (1999) 33–53.
- [14] I. Safarik, L. Ptackova, M. Safarikova, *Biotechnol. Lett.* 23 (2001) 1953–1956.
- [15] E. Palecek, Ph.D. Thesis, Masaryk University, Brno, 1959.
- [16] E. Palecek, *Nature* 188 (1960) 656–657.
- [17] E. Palecek, in: J.N. Davidson, W.E. Cohn (Eds.), *Progress in Nucleic Acid Research and Molecular Biology*, vol. 9, Academic Press, New York, 1969, pp. 31–73.
- [18] E. Palecek, *Anal. Biochem.* 108 (1980) 129–138.
- [19] E. Palecek, *Naturwiss* 45 (1958) 186–187.
- [20] E. Palecek, *Coll. Czech. Chem. Commun.* 25 (1960) 2283–2289.
- [21] E. Palecek, in: L. Grossman, K. Moldave (Eds.), *Methods in Enzymology: Nucleic Acids*, Part D, vol. 21, Academic Press, New York, 1971, pp. 3–24.
- [22] E. Palecek, *J. Mol. Biol.* 20 (1966) 263–281.
- [23] E. Palecek, B.D. Fray, *Arch. Biochem. Biophys.* 115 (1966) 431–436.
- [24] E. Palecek, *Electroanalysis* 8 (1996) 7–14.
- [25] E. Lukášová, E. Palecek, *Radiat. Res.* 47 (1971) 51–65.
- [26] E. Palecek, *Prog. Nucleic Acid Res. Mol. Biol.* 18 (1976) 151–213.
- [27] V. Brabec, *Bioelectrochem. Bioenerg.* 8 (1981) 437–449.
- [28] E. Palecek, *Talanta* 56 (2002) 809–819.
- [29] E. Palecek, M. Fojta, F. Jelen, V. Vetterl, in: A.J. Bard, M. Stratsmann (Eds.), *Bioelectrochemistry, The Encyclopedia of Electrochemistry*, vol. 9, Wiley-VCH, Weinheim, 2002, pp. 365–429.
- [30] E. Palecek, F. Jelen, in: E. Palecek, F. Scheller, J. Wang (Eds.), *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005, pp. 74–174.
- [31] J. Wang, in: E. Palecek, F. Scheller, J. Wang (Eds.), *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005, pp. 369–384.
- [32] J. Wang, in: E. Palecek, F. Scheller, J. Wang (Eds.), *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005, pp. 175–194.
- [33] J. Wang, G. Rivas, X. Cai, E. Palecek, P. Nielsen, H. Shiraishi, N. Dontha, D. Luo, C. Parrado, M. Chicharro, P.A.M. Farias, F.S. Valera, D.H. Grant, M. Ozsoz, M.N. Flair, *Anal. Chim. Acta* 347 (1997) 1–8.
- [34] T.G. Drummond, M.G. Hill, J.K. Barton, *Nat. Biotechnol.* 21 (2003) 1192–1199.
- [35] M.J. Tarlov, A.B. Steel, in: J.F. Rusling (Ed.), *Biomolecular Films. Design, Function, and Applications*, Marcel Dekker, New York, 2003, pp. 545–608.
- [36] H.H. Thorp, *Top. Curr. Chem.* 237 (2004) 159–181.
- [37] M.I. Pividori, S. Alegret, *Anal. Lett.* 38 (2005) 2541–2565.
- [38] J. Labuda, M. Fojta, F. Jelen, E. Palecek, in: C.A. Grimes, E.C. Dickey, M.V. Pishko (Eds.), *Encyclopedia of Sensors*, vol. 3 E–F, American Scientific Publishers, Stevenson Ranch, CA, USA, 2006, pp. 201–228.
- [39] V. Ostatna, E. Palecek, *Langmuir* 22 (2006) 6481–6484.
- [40] M. Fojta, V. Vetterl, M. Tomschik, F. Jelen, P. Nielsen, J. Wang, E. Palecek, *Biophys. J.* 72 (1997) 2285–2293.
- [41] P. Singhal, W.G. Kuhr, *Anal. Chem.* 69 (1997) 3552–3557.
- [42] E. Palecek, F. Jelen, M.A. Hung, J. Lasovsky, *Bioelectrochem. Bioenerg.* 8 (1981) 621–631.
- [43] E. Palecek, J. Osteryoung, R.A. Osteryoung, *Anal. Chem.* 54 (1982) 1389–1394.
- [44] E. Palecek, *Anal. Chim. Acta* 174 (1985) 103–113.
- [45] M.M. Correia dosSantos, C.M.L.F. Lopes, M.L.S. Goncalves, *Bioelectrochem. Bioenerg.* 39 (1996) 55–60.
- [46] P.A.M. Farias, A.D. Wagener, A.A. Castro, *Talanta* 55 (2001) 281–290.
- [47] S. Hason, V. Vetterl, *Anal. Chem.* 78 (2006) 5179–5183.
- [48] S. Hason, V. Vetterl, *Talanta* 69 (2006) 572–580.
- [49] F. Jelen, B. Yosypchuk, A. Kourilova, L. Novotny, E. Palecek, *Anal. Chem.* 74 (2002) 4788–4793.
- [50] J. Wang, X. Cai, J.Y. Wang, C. Jonsson, E. Palecek, *Anal. Chem.* 67 (1995) 4065–4070.
- [51] A.M.O. Brett, J.A.P. Piedade, L.A. Silva, V.C. Dicuilescu, *Anal. Biochem.* 332 (2004) 321–329.
- [52] X. Cai, G. Rivas, P.A.M. Farias, H. Shiraishi, J. Wang, M. Fojta, E. Palecek, *Bioelectrochem. Bioenerg.* 401 (1996) 41–47.

- [53] X.H. Cai, G. Rivas, P.A.M. Farias, H. Shiraishi, J. Wang, E. Paleček, *Anal. Chim. Acta* 332 (1996) 49–57.
- [54] X. Cai, G. Rivas, P.A.M. Farias, H. Shiraishi, J. Wang, E. Paleček, *Electroanalysis* 8 (1996) 753–758.
- [55] X. Cai, G. Rivas, H. Shiraishi, P. Farias, J. Wang, M. Tomshik, F. Jelen, E. Paleček, *Anal. Chim. Acta* 344 (1997) 65–76.
- [56] M. Fojta, L. Havran, E. Paleček, *Electroanalysis* 9 (1997) 1033–1034.
- [57] E. Paleček, M. Fojta, M. Tomschik, J. Wang, *Biosens. Bioelectron.* 13 (1998) 621–628.
- [58] E. Paleček, F. Scheller, J. Wang, *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005.
- [59] J. Wang, M. Jiang, E. Paleček, *Bioelectrochem. Bioenerg.* 48 (1999) 477–480.
- [60] J. Wang, M. Ozsoz, X. Cai, G. Rivas, H. Shiraishi, D.H. Grant, M. Chicharro, J. Fernandes, E. Paleček, *Bioelectrochem. Bioenerg.* 45 (1998) 33–40.
- [61] J. Wang, E. Paleček, P. Nielsen, G. Rivas, X. Cai, H. Shiraishi, H. Dontha, D. Luo, P.A.M. Farias, *J. Am. Chem. Soc.* 118 (1996) 7667.
- [62] J. Wang, G. Rivas, X. Cai, M. Chicharro, N. Dontha, D. Luo, E. Paleček, P.E. Nielsen, *Electroanalysis* 9 (1997) 120–124.
- [63] J. Wang, G. Rivas, X. Cai, M. Chicharro, C. Parrado, N. Dontha, A. Begleiter, M. Mowat, E. Paleček, P.E. Nielsen, *Anal. Chim. Acta* 344 (1997) 111–118.
- [64] J. Wang, G. Rivas, X. Cai, M. Chicharro, P.A.M. Farias, E. Paleček, *Electroanalysis* 8 (1996) 902–906.
- [65] E. Paleček, V. Vetterl, *Biopolymers* 6 (1968) 917–928.
- [66] M. Tomschik, L. Havran, M. Fojta, E. Paleček, *Electroanalysis* 10 (1998) 403–409.
- [67] M. Fojta, R.P. Bowater, V. Stanková, L. Havran, D.M.J. Lilley, E. Paleček, *Biochemistry* 37 (1998) 4853–4862.
- [68] M. Fojta, E. Paleček, *Anal. Chim. Acta* 342 (1997) 1–12.
- [69] E. Paleček, M. Fojta, *Anal. Chem.* 66 (1994) 1566–1571.
- [70] M. Fojta, R. Doffkova, E. Paleček, *Electroanalysis* 8 (1996) 420–426.
- [71] E.E. Ferapontova, E. Dominguez, *Electroanalysis* 15 (2003) 629–634.
- [72] E. Paleček, M.A. Hung, *Anal. Biochem.* 132 (1983) 236–242.
- [73] E. Lukasova, M. Vojtiskova, F. Jelen, T. Sticzay, E. Paleček, *Gen. Physiol. Biophys.* 3 (1984) 175–191.
- [74] E. Lukasova, F. Jelen, E. Paleček, *Gen. Physiol. Biophys.* 1 (1982) 53–70.
- [75] E. Paleček, M. Vojtiskova, F. Jelen, E. Lukasova, *Bioelectrochem. Bioenerg.* 12 (1984) 135–136.
- [76] E. Paleček, in: J.N. Abelson, M.I. Simon (Eds.), *Methods in Enzymology*, vol. 212, Academic Press, New York, 1992, pp. 139–155.
- [77] F. Jelen, P. Karlovsky, P. Pecinka, E. Makaturova, E. Paleček, *Gen. Physiol. Biophys.* 10 (1991) 461–473.
- [78] B. Yosypchuk, M. Fojta, L. Havran, M. Heyrovsky, E. Paleček, *Electroanalysis* 18 (2006) 186–194.
- [79] R. Kizek, L. Havran, M. Fojta, E. Paleček, *Bioelectrochemistry* 55 (2002) 119–121.
- [80] M. Fojta, L. Havran, S. Billova, P. Kostecka, M. Masarik, R. Kizek, *Electroanalysis* 15 (2003) 431–440.
- [81] L. Havran, M. Fojta, E. Paleček, *Bioelectrochemistry* 63 (2004) 239–243.
- [82] M. Fojta, L. Havran, R. Kizek, S. Billova, *Talanta* 56 (2002) 867–874.
- [83] M. Fojta, L. Havran, R. Kizek, S. Billova, E. Paleček, *Biosens. Bioelectron.* 20 (2004) 985–994.
- [84] M. Fojta, P. Kostecka, M. Trefulka, L. Havran, E. Paleček, *Anal. Chem.* 79 (2007) 1022–1029.
- [85] G.U. Flechsig, T. Reske, *Anal. Chem.* 79 (2007) 2125–2130.
- [86] M. Trefulka, N. Ferreyra, V. Ostatna, M. Fojta, G. Rivas, E. Paleček, *Electroanalysis* 19 (2007) 1334–1338.
- [87] M. Trefulka, V. Ostatna, L. Havran, M. Fojta, E. Paleček, *Electroanalysis* 19 (2007) 1281–1287.
- [88] C. Fan, K.W. Plaxco, A.J. Heeger, *Proc. Nat. Acad. Sci. U.S.A.* 100 (2003) 9134–9137.
- [89] A. Anne, A. Bouchardon, J. Moiroux, *J. Am. Chem. Soc.* 125 (2003) 1112–1113.
- [90] A.J. Baca, F.M. Zhou, J. Wang, J.B. Hu, J.H. Li, J.X. Wang, Z.S. Chikneyan, *Electroanalysis* 16 (2004) 73–80.
- [91] J.M. Gibbs, S.J. Park, D.R. Anderson, K.J. Watson, C.A. Mirkin, S.T. Nguyen, *J. Am. Chem. Soc.* 127 (2005) 1170–1178.
- [92] T. Ihara, Y. Maruo, S. Takanaka, M. Takagi, *Nucleic Acids Res.* 24 (1996) 4273–4280.
- [93] C.E. Immoos, S.J. Lee, M.W. Grinstaff, *J. Am. Chem. Soc.* 126 (2004) 10814–10815.
- [94] N.D. Popovich, S.S. Wong, B.K.H. Yen, H.Y. Yeom, D.C. Paine, *Anal. Chem.* 74 (2002) 3127–3133.
- [95] S. Sato, T. Nojima, S. Takenaka, *J. Organometal. Chem.* 689 (2004) 4722–4728.
- [96] C.J. Yu, H. Yowanto, Y.J. Wan, T.J. Meade, Y. Chong, M. Strong, L.H. Donilon, J.F. Kayyem, M. Gozin, G.F. Blackburn, *J. Am. Chem. Soc.* 122 (2000) 6767–6768.
- [97] C.J. Yu, Y.J. Wan, H. Yowanto, J. Li, C.L. Tao, M.D. James, C.L. Tan, G.F. Blackburn, T.J. Meade, *J. Am. Chem. Soc.* 123 (2001) 11155–11161.
- [98] M. Hocek, P. Stepnicka, J. Ludvik, I. Cisarova, I. Votruba, D. Reha, P. Hobza, *Chem. Eur. J.* 10 (2004) 2058–2066.
- [99] T. Ihara, M. Nakayama, M. Murata, K. Nakano, M. Maeda, *Chem. Commun.* 17 (1997) 1609–1610.
- [100] S.O. Kelley, E.M. Boon, J.K. Barton, N.M. Jackson, M.G. Hill, *Nucleic Acid Res.* 27 (1999) 4830–4837.
- [101] Y.D. Mao, C.X. Luo, Q. Ouyang, *Nucleic Acid Res.* 31 (2003) e108.
- [102] F. Patolsky, Y. Weizmann, I. Willner, *J. Am. Chem. Soc.* 124 (2002) 770–772.
- [103] E. Paleček, M. Fojta, *Anal. Chem.* 73 (2001) 74A–83A.
- [104] P.E. Nielsen, M. Egholm, O. Buchardt, *Bioconjugate Chem.* 5 (1994) 3–7.
- [105] J. Wang, G. Rivas, X.H. Cai, M. Chicharro, C. Parrado, N. Dontha, A. Begleiter, M. Mowat, E. Paleček, P.E. Nielsen, *Anal. Chim. Acta* 344 (1997) 111–118.
- [106] M. Tomschik, F. Jelen, L. Havran, L. Trnkova, P.E. Nielsen, E. Paleček, *J. Electroanal. Chem.* 476 (1999) 71–80.
- [107] M. Steichen, Y. Decrem, E. Godfroid, C. Buess-Herman, *Biosens. Bioelectron.* 22 (2007) 2237–2243.
- [108] B.R. Baker, R.Y. Lai, M.S. Wood, E.H. Doctor, A.J. Heeger, K.W. Plaxco, *J. Am. Chem. Soc.* 128 (2006) 3138–3139.
- [109] E. Paleček, *Trends Biotechnol.* 22 (2004) 55–58.
- [110] E. Paleček, S. Billova, L. Havran, R. Kizek, A. Miculkova, F. Jelen, *Talanta* 56 (2002) 919–930.
- [111] A. Erdem, M.I. Pividori, A. Lermo, A. Bonanni, M. del Valle, S. Alegret, *Sens. Actuators B-Chem.* 114 (2006) 591–598.
- [112] M. Vrábel, M. Hocek, L. Havran, M. Fojta, I. Votruba, B. Klepetářová, R. Pohl, L. Rulíšek, L. Zendlová, P. Hobza, I. Shih, E. Mabery, R. Mackman, *Eur. J. Inorg. Chem.* 2007 (2007) 1752–1769.
- [113] E. Paleček, R. Kizek, L. Havran, S. Billova, M. Fojta, *Anal. Chim. Acta* 469 (2002) 73–83.
- [114] J. Wang, G.D. Liu, M.R. Jan, *J. Am. Chem. Soc.* 126 (2004) 3010–3011.
- [115] M. Fojta, L. Havran, M. Vojtisková, E. Paleček, *J. Am. Chem. Soc.* 126 (2004) 6532–6533.
- [116] J. Wang, D.K. Xu, A. Erdem, R. Polsky, M.A. Salazar, *Talanta* 56 (2002) 931–938.
- [117] J. Wang, A.N. Kawde, M. Musameh, G. Rivas, *Analyst* 127 (2002) 1279–1282.
- [118] A.A. Garcia, A. Egatz-Gomez, S.A. Lindsay, P. Dominguez-Garcia, S. Melle, M. Marquez, M.A. Rubio, S.T. Picraux, D.Q. Yang, P. Aella, M.A. Hayes, D. Gust, S. Loyprasert, T. Vazquez-Alvarez, J. Wang, *J. Magn. Mater.* 311 (2007) 238–243.
- [119] M.J. LaGier, C.A. Scholin, J.W. Fell, J. Wang, K.D. Goodwin, *Mar. Pollut. Bull.* 50 (2005) 1251–1261.
- [120] S. Lindsay, T. Vazquez, A. Egatz-Gomez, S. Loyprasert, A.A. Garcia, *J. Wang, Analyst* 132 (2007) 412–416.
- [121] J. Wang, M. Musameh, R. Laocharoensuk, O. Gonzalez-Garcia, J. Oni, D. Gervasio, *Electrochem. Commun.* 8 (2006) 1106–1110.
- [122] J. Wang, M. Scampicchio, R. Laocharoensuk, F. Valentini, O. Gonzalez-Garcia, J. Burdick, *J. Am. Chem. Soc.* 128 (2006) 4562–4563.
- [123] J. Wang, G.D. Liu, M.R. Jan, Q.Y. Zhu, *Electrochem. Commun.* 5 (2003) 1000–1004.
- [124] J. Wang, *Electroanalysis* 19 (2007) 769–776.

- [125] B. Yosypchuk, M. Heyrovsky, E. Palecek, L. Novotny, *Electroanalysis* 14 (2002) 1488–1493.
- [126] R. Fadrna, B. Yosypchuk, M. Fojta, T. Navrátil, L. Novotny, *Anal. Lett.* 37 (2004) 399–413.
- [127] J. Wang, A.B. Kawde, *Analyst* 127 (2002) 383–386.
- [128] J. Wang, D.K. Xu, A.N. Kawde, R. Polsky, *Anal. Chem.* 73 (2001) 5576–5581.
- [129] J. Wang, G.D. Liu, R. Polsky, A. Merkoci, *Electrochem. Commun.* 4 (2002) 722–726.
- [130] S. Centi, S. Tombelli, M. Minunni, M. Mascini, *Anal. Chem.* 79 (2007) 1466–1473.
- [131] J. Wang, O. Rincon, R. Polsky, E. Dominguez, *Electrochem. Commun.* 5 (2003) 83–86.
- [132] M. Pumera, M.T. Castaneda, M.I. Pividori, R. Eritja, A. Merkoci, S. Alegret, *Langmuir* 21 (2005) 9625–9629.
- [133] M.I. Pividori, A. Lermo, S. Hernandez, J. Barbe, S. Alegret, S. Campoy, *Afinidad* 62 (2006) 13–18.
- [134] E. Katz, B. Willner, I. Willner, in: E. Palecek, F. Scheller, J. Wang (Eds.), *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005, pp. 195–246.
- [135] E. Katz, I. Willner, J. Wang, *Electroanalysis* 16 (2004) 19–44.
- [136] E. Katz, L. Sheeney-Haj-Ichia, I. Willner, *Chem.-Eur. J.* 8 (2002) 4138–4148.
- [137] M.Y. Han, X.H. Gao, J.Z. Su, S. Nie, *Nat. Biotechnol.* (2001) 631–635.
- [138] I. Willner, F. Patolsky, J. Wasserman, *Angew. Chem.-Int. Ed.* 40 (2001) 1861–1864.
- [139] J. Wang, G. Liu, A. Merkoci, *J. Am. Chem. Soc.* 125 (2003) 3214–3215.
- [140] J. Wang, G.D. Liu, G. Rivas, *Anal. Chem.* 75 (2003) 4667–4671.
- [141] J. Wang, G.D. Liu, Q.Y. Zhu, *Anal. Chem.* 75 (2003) 6218–6222.
- [142] J. Wang, R. Polsky, A. Merkoci, K.L. Turner, *Langmuir* 19 (2003) 989–991.
- [143] A.N. Kawde, J. Wang, *Electroanalysis* 16 (2004) 101–107.
- [144] W.J. Miao, A.J. Bard, *Anal. Chem.* 76 (2004) 5379–5386.
- [145] L. Alfonta, A.K. Singh, I. Willner, *Anal. Chem.* 73 (2001) 91–102.
- [146] J. Wang, J.H. Li, A.J. Baca, J.B. Hu, F.M. Zhou, W. Yan, D.W. Pang, *Anal. Chem.* 75 (2003) 3941–3945.
- [147] E. Katz, I. Willner, *Chemphyschem* 5 (2004) 1085–1104.
- [148] R.H. Baughman, A.A. Zakhidov, W.A. de Heer, *Science* 297 (2002) 787–792.
- [149] M.L. Pedano, G.A. Rivas, *Electrochem. Commun.* 6 (2004) 10–16.
- [150] J. Wang, A.N. Kawde, M. Musameh, *Analyst* 128 (2003) 912–916.
- [151] J. Li, H.T. Ng, A. Cassell, W. Fan, H. Chen, Q. Ye, J. Koehne, J. Han, M. Meyyappan, *Nano Lett.* 3 (2003) 597–602.
- [152] M. Fojta, P. Brazdilova, K. Cahova, P. Pecinka, *Electroanalysis* 18 (2006) 141–151.
- [153] D.J. Caruana, A. Heller, *J. Am. Chem. Soc.* 121 (1999) 769–774.
- [154] F. Azek, C. Grossiord, M. Joannes, B. Limoges, P. Brossier, *Anal. Biochem.* 284 (2000) 107–113.
- [155] E. Zacco, M.I. Pividori, S. Alegret, *Anal. Chem.* 78 (2006) 1780–1788.
- [156] A. Warsinke, W. Stocklein, E. Leupold, E. Micheel, F. Scheller, in: E. Palecek, F. Scheller, J. Wang (Eds.), *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005, pp. 451–483.
- [157] A. Kuderová-Krejčová, A.M. Poverenny, E. Palecek, *Nucleic Acid Res.* 19 (1991) 6811–6817.
- [158] E. Palecek, in: A. Townshend (Ed.), *Encyclopedia of Analytical Science*, vol. 6, Academic Press, London, 1995, pp. 3600–3609.
- [159] V. Campuzano, L. Montermini, M.D. Molto, L. Pianese, M. Cossee, F. Cavalcanti, E. Monros, F. Rodius, F. Duclos, A. Monticelli, et al., *Science* 271 (1996) 1423–1427.
- [160] S.A. Brazill, P.H. Kim, W.G. Kuhr, *Anal. Chem.* 73 (2001) 4882–4890.
- [161] D.A. Di Giusto, W.A. Wlasoff, S. Giesebrecht, J.J. Gooding, G.C. King, *J. Am. Chem. Soc.* 126 (2004) 4120–4121.
- [162] H. Weizman, Y. Tor, *J. Am. Chem. Soc.* 124 (2002) 1568–1569.
- [163] E. Palecek, in: E. Palecek, F. Scheller, J. Wang (Eds.), *Electrochemistry of Nucleic Acids and Proteins. Towards Electrochemical Sensors for Genomics and Proteomics*, Elsevier, Amsterdam, 2005, pp. 690–750.
- [164] E. Palecek, M. Masarik, K.R.D. Kuhlmeier, J. Hassmann, J. Schulein, *Anal. Chem.* 76 (2004) 5930–5936.
- [165] M. Masarik, A. Stobiecka, R. Kizek, F. Jelen, Z. Pechan, W. Hoyer, T.M. Jovin, V. Subramaniam, E. Palecek, *Electroanalysis* 16 (2004) 1172–1181.
- [166] M. Masarik, K. Cahova, R. Kizek, E. Palecek, M. Fojta, *Anal. Bioanal. Chem.* 388 (2007) 259–270.
- [167] J.G. Bruno, J.L. Kiel, *Biotechniques* 32 (2002) 178–183.
- [168] J.K. Herr, J.E. Smith, C.D. Medley, D.H. Shangguan, W.H. Tan, *Anal. Chem.* 78 (2006) 2918–2924.
- [169] A.N. Kawde, M.C. Rodriguez, T.M.H. Lee, J. Wang, *Electrochem. Commun.* 7 (2005) 537–540.
- [170] M.B. Murphy, S.T. Fuller, P.M. Richardson, S.A. Doyle, *Nucleic Acids Res.* 31 (2003).
- [171] H.A. Oktem, G. Bayramoglu, V.C. Ozalp, M.Y. Arica, *Biotechnol. Progr.* 23 (2007) 146–154.
- [172] J.A. Hansen, J. Wang, A.N. Kawde, Y. Xiang, K.V. Gothelf, G. Collins, *J. Am. Chem. Soc.* 128 (2006) 2228–2229.
- [173] J. Wang, G.D. Liu, B. Munge, L. Lin, Q.Y. Zhu, *Angew. Chem. Int. Ed.* 43 (2004) 2158–2161.
- [174] Z.H. Fan, S. Mangru, R. Granzow, P. Heaney, W. Ho, Q. Dong, R. Kumar, *Anal. Chem.* 71 (1999) 4851–4859.

Carbon nanofiber vs. carbon microparticles as modifiers of glassy carbon and gold electrodes applied in electrochemical sensing of NADH

Briza Pérez^{a,b}, Manel del Valle^b, Salvador Alegret^b, Arben Merkoçi^{a,b,*}

^a *Nanobioelectronics & Biosensors Group, Institut Català de Nanotecnologia,
Campus Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain*

^b *Grup de Sensors & Biosensors, Departament de Química, Universitat Autònoma de Barcelona,
08193 Bellaterra, Catalonia, Spain*

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Abstract

Carbon materials (CMs), such as carbon nanotubes (CNTs), carbon nanofibers (CNFs), and carbon microparticles (CMPs) are used as doping materials for electrochemical sensors. The efficiency of these materials (either before or after acidic treatments) while being used as electrocatalysts in electrochemical sensors is discussed for β -nicotinamide adenine dinucleotide (NADH) detection using cyclic voltammetry (CV). The sensitivity of the electrodes (glassy carbon (GC) and gold (Au)) modified with both treated and untreated materials have been deeply studied. The response efficiencies of the GC and Au electrodes modified with CNF and CMP, using dimethylformamide (DMF) as dispersing agent are significantly different due to the peculiar physical and chemical characteristics of each doping material. Several differences between the electrocatalytic activities of CMs modified electrodes upon NADH oxidation have been observed. The CNF film promotes better the electron transfer of NADH minimizing the oxidation potential at +0.352 V. Moreover higher currents for the NADH oxidation peak have been observed for these electrodes. The shown differences in the electrochemical reactivities of CNF and CMP modified electrodes should be with interest for future applications in biosensors.

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1. Introduction

Carbon materials (CMs), such as graphite/carbon microparticles (CMPs), carbon nanotubes (CNTs), and carbon nanofibers (CNFs) have shown significant improvements in the development of novel sensors and biosensors [1–5]. High-surface area of CMs may also lead to new signal transduction processes [6,7] and to increased sensitivity in sensing applications [8–10].

Various processes used to be involved within a (bio)sensor system. These ranges from the analyte recognition, catalytic conversion, oxidation–reduction processes, mediation of electron

transfer, and finally the signal transduction and data display. To achieve a smooth running of the above processes a careful evaluation of the properties of the materials, ensuring an efficiency of their electrochemical properties, while incorporating them into the biosensor membranes must be considered. A better interaction and communication between the biomolecule and the transducer is also aimed to be achieved while using these materials. Only under these conditions a biosensor device will provide analytically useful information.

In recent years, with the great progress made in nanoscience and nanotechnology, interest is increasing in exploring the unique properties and potential technological applications of various nanostructures [11,12]. Many nanomaterials, such as peptide nanotubes [11], poly(1,2-diaminobenzene) nanotubules [13], and TiO₂ nanostructured films [14], particularly carbon nanotubes (CNT) [15–17], have been devoted to decreasing the high-overpotential for β -nicotinamide adenine dinucleotide

* Corresponding author at: Nanobioelectronics & Biosensors Group, Institut Català de Nanotecnologia, Campus Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain.

E-mail address: arben.merkoci.icn@uab.es (A. Merkoçi).

(NADH) oxidation, minimizing surface fouling, and improving electron-transfer kinetics. Depending on the size, shape, and internal structure, nanoparticles frequently display unique physical and chemical properties [18].

CMP represents a highly ordered form of carbon. It is a solid that possesses delocalized π electrons on the basal planes, and this property imparts a weak basic character to the material in which the polarity can be manipulated by selected chemical treatments [19]. Carbon nanofibers (CNFs) represent carbon fibers with a nanometer-size diameter and no hollow core, but with many edge sites on the outer wall [20]. It has been recognized as one of the very promising materials based on its nanostructure and particular properties [21] and is expected to be used in various applications such as catalysts or catalyst supports [22], probe tips [23], and fuel cells [24].

Although there is a large amount of size overlap between CNT and CNF, there are significant structural differences. CNF consist of graphitic layers that stack either at an angle, perpendicular or parallel to the tube axis [25–28] while the CNT represent two single-walled and multi-walled structures. Single-wall CNTs (SWCNTs) comprise of a cylindrical graphite sheet of nanoscale diameter capped by hemispherical ends. The multi-wall CNTs (MWCNTs) comprise several to tens of incommensurate concentric cylinders of these graphitic shells with a layer spacing of 0.3–0.4 nm. MWCNTs tend to have diameters in the range 2–100 nm. The MWCNT can be considered as a mesoscale graphite system, whereas the SWCNT is truly a single large molecule [5].

Due to the unique characteristics of CNF there is a wide range of applications. The thermal and chemical stability, high-surface area, low-ohmic resistance, and the surface properties are the unique qualities that are exploited when CNF are used as catalytic support materials [25–30]. The surface at the tip of a CNF possesses a large amount of exposed edges due to the arrangement of the graphitic layers [31].

Recently, Wu et al. [32] studied the excellent catalytic activity of soluble CNF with good dispersion, to the oxidation of NADH for biosensing application. They show as the edge plane sites and oxygen-rich groups presented on the CNF surface could be partially responsible for its electrocatalytic behavior, which induced a substantial decrease by 573 mV in the overpotential of NADH oxidation reaction (compared to a bare glassy carbon electrode).

CNFs lead to more facile electron transfer [33] compared to CMPs. The solubility of CNF in dimethylformamide (DMF) facilitates its manipulation, including the coating on electrode surfaces for electrochemical biosensing applications, because the insolubility of carbon materials in most solvents is a major obstacle in implementing their widespread use [34].

In this work, chemically treated and untreated carbon materials (CNF and CMP) were used as modifiers of glassy carbon and gold electrode surfaces. The objective was to compare their efficiencies for electrochemical oxidation of NADH while using cyclic voltammetry (CV). Interesting results with interest for future biosensing applications will be shown.

2. Experimental

2.1. Materials and reagents

Herringbone graphite nanofibers, CNFs (100 nm in width and up to 10 μ in length) were purchased from catalytic materials. Graphite powder (carbon microparticle, CMPs, size 50 μ m) was obtained from BDH, UK. Further purification of carbon material was accomplished by stirring the CMs in 2 M nitric acid (PanReac, Spain) at 25 °C for 24 h [35]. A 0.7 mg of carbon material (CMP or CNF) was dispersed in 700 μ L of dimethylformamide (DMF, from Sigma–Aldrich). β -Nicotinamide adenine dinucleotide reduced form (NADH), potassium dihydrogen phosphate, potassium hydrogen phosphate, and sodium hydroxide were obtained from Sigma–Aldrich (Spain). The working solutions (NADH) were prepared daily by dilution in 0.1 M phosphate buffer at pH 7.0 with ultra pure water from a Millipore-MilliQ system.

2.2. Apparatus

Scanning electron microscopy (SEM, Hitachi S-570, Tokyo, Japan) was used to study the morphology of the electrode surfaces. Transmission electron microscope (TEM) images of the carbon materials were obtained using a Hitachi H-7000 (Hitachi Ltd., Tokyo, Japan). Scanning electron microscopy JEOL JSM-6300 (SEM, JEOL Ltd., Tokyo, Japan) with microanalysis equipped by an X-ray energy dispersive spectrometer (EDS) (Link ISIS-200, England) was used in order to evaluate the amount of chemical impurities (remained from their synthesis) and of oxygen groups introduced during the treatment of CMs with HNO_3 .

The electrochemical measurements were performed with an electrochemical analyzer CHI 630B (CH Instruments) connected to a personal computer. The measurements were carried with a typical cell of 10 mL at room temperature (25 °C), using a three-electrode configuration. A platinum electrode and Ag/AgCl were used as counter and reference electrode, respectively. The glassy carbon (GC) and gold (Au) electrodes, with diameter of 2 mm, were from CH Instruments (Austin, TX, USA). The GC or Au modified electrodes using CNFs or CMPs suspended in DMF were also used as working electrodes. Modified electrodes were prepared in our laboratory according to the procedure described below.

2.3. Modification of GC and Au electrode surfaces

CNF and CMP were purified prior to use by a 2 M nitric acid solution. The bare electrodes (GC and Au electrodes) were polished with alumina paper (polishing strips 301044-001, Orion, Spain) and washed with water. These electrodes were then used for further modifications with CMs dispersed into DMF. Usually 0.7 mg CMs (treated or untreated) were mixed/dispersed into 700 μ L of DMF by ultrasonic agitation for about 1 h. After, 10 μ L of CMs–DMF solution were dropped directly onto of GC and Au electrode surfaces and allowed to dry at 40 °C for 45 min. Finally the electrodes were washed with water, and were ready

for use. For comparison experiments, bare GC and Au electrodes were prepared, following exactly the same experimental procedure.

2.4. Electrochemical measurements

Electrochemical experiments were performed using a three-electrode system with an Ag/AgCl reference electrode, a Pt wire auxiliary electrode and CNF/GC, CMP/GC or CNF/Au, CMP/Au as working electrodes.

The measurements were performed with an electrochemical analyzer CHI 630B (CH Instruments) connected to a personal computer. Cyclic voltammograms using electrodes modified with treated and untreated CMs were performed in 2.5 mM NADH solution. The response to NADH was measured in 10 mL of a 0.1 M phosphate buffer solution (PBS) pH 7.0.

The potential range used for NADH determination was -0.6 to $+1.2$ V for CNF-DMF/GC, CMP-DMF/GC, CNF-DMF/Au, and CMP-DMF/Au. The background current was allowed to decay to a constant level before aliquots of NADH sample were added to the buffer solution.

3. Results and discussion

3.1. Characteristics of the carbon materials and the corresponding films

The dispersion of the CNF-DMF or CMP-DMF onto the electrode surfaces (GC and Au) is an important issue in producing carbon films onto the electrode surfaces. Scanning electron microscopy was used to analyze the carbon films on GC and Au electrode surfaces. Fig. 1 compares SEM images of treated CNF and CMP and untreated CNF and CMP films deposited onto the surfaces of GC and Au electrodes. It can be appreciated that the treated CMPs produce a more uniform carbon film over the electrode surfaces (GC and Au) compared to untreated CMPs. The CMPs are more scattered over the gold electrode surface than in the GC electrode. However, untreated CNF create a better uniform/homogeneous film and even more coverage over the surface of GC and Au electrodes, while treated CNF creates more scattered and even thinner layer onto the GC and Au surfaces. Fig. 1 shows that in all the cases, the surfaces of the electrodes are covered (although not in the same level) with either the treated or with untreated CMs which seem to be available to act as an electrochemical sensing surface for NADH detection.

TEM images reveal the structure of the CMs, both CMPs and CNFs (Fig. 2). CMPs structures consists of carbon layers (in which the carbon atoms are arranged in an open honeycomb network) situated ones over others and the electrical conductivity between them is very poor [36]. The impurity species may occupy some interstitial position between the graphene layer planes, which are bonded by a weak van der Waals force. These weak interplanar bonds at graphite allow dopant atoms or molecules to be intercalated between the carbon layers to form intercalation compounds [36]. The TEM of CNFs are also presented. A clear difference of TEMs between CMPs and CNFs

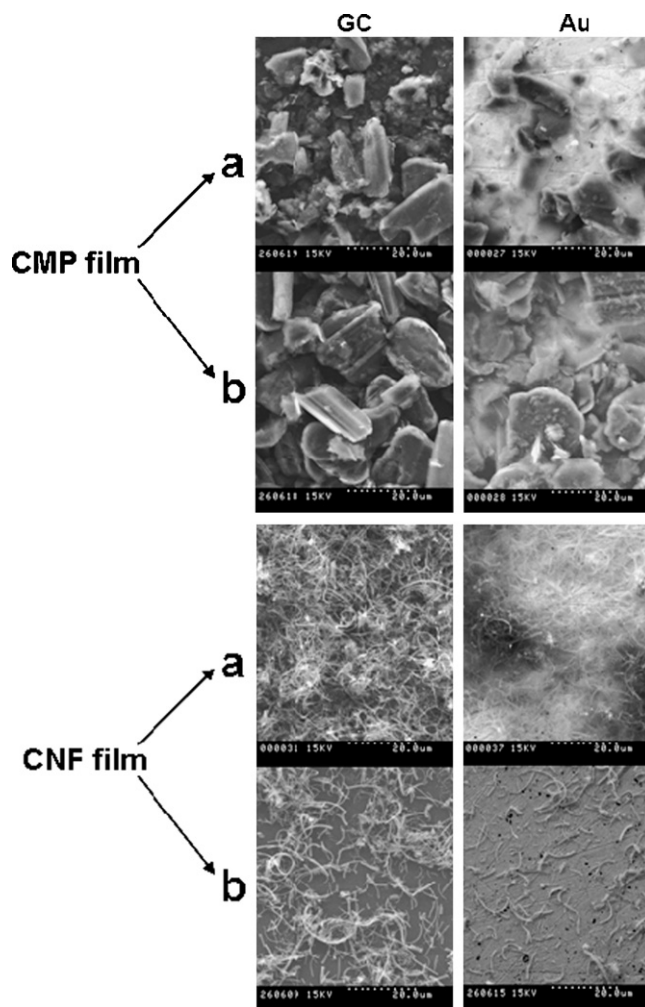


Fig. 1. SEM images for CMP and CNF films on GC and Au electrode surfaces; without treatment (a) and with treatment (b) in HNO_3 . The same acceleration voltage (5 kV) and resolution are used.

can be observed while the differences between the treated and untreated CMs are not visible except of a kind of structure rupture after treatment with HNO_3 of both materials. In addition we can observe a slight growth of the surface area covered with treated CNFs.

The measured surface area can be regarded as the external surface area of the fibers.

Apparently, with the process of oxidation in $\text{HNO}_3/\text{H}_2\text{SO}_4$ or KMnO_4/H^+ , the macrostructure of the fibers becomes somewhat less dense, and a slightly higher pore volume (space between the fibers). As a result of the oxidation, the fibers have a surface roughening that can explain the increase in surface area (see Fig. 2b CNF), but its pore volume and the average pore diameter are decreased [37]. Probably, as a result of this decrease, the electronic transference is smaller in treated CNF, and due to its porous structure in comparison with the structure of the CMPs that is more rigid the electrochemical response is better with untreated CNF.

Fig. 3 is a schematic of CMP and CNF structures showing the graphene layers along with several possibilities for other molecules (NADH in our case) to enter in between. CNF struc-

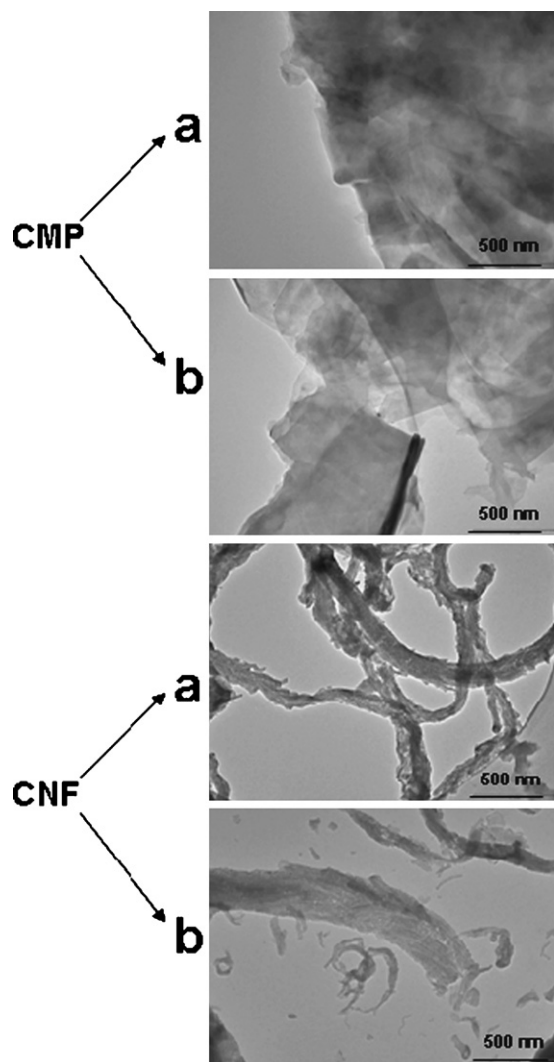


Fig. 2. TEM images of CMPs and carbon nanofibers (CNFs) untreated (a) and treated (b) with HNO_3 , and suspended using dimethylformamide (DMF) as described in the text.

ture is of the herringbone type disclosing the origin of the surface roughness. Probably, CNF due to its structure can adsorb species on their external and internal surfaces and between adjacent nanofibers. In the case of CNF, the guest species is believed to decorate the exterior of the nanofiber, and also to enter the hollow cores of the CNF.

Moreover, the two types of carbon materials have a difference in orientation of the carbon planes. Taking into consideration the textures of CMs it can be previewed how an oxidant (for example HNO_3) might introduce changes at the structures. Fig. 3 presents the possible sites of attachment that can be introduced onto the surface of the carbon materials studied.

The SEM microanalysis and X-ray energy dispersive spectrometer (EDS) results for CNFs and CMPs, comparing the CMs before and after treatment with HNO_3 (results now shown) could give also an insight of the chemical impurities and the oxygen groups present in each CMs. Table 1 represents the microanalysis data obtained by SEM of the untreated and treated carbon materials. These data show that there are variations between the

Table 1

Microanalysis data of the CMP and CNF (without any treatment and with treatment with HNO_3) obtained by SEM (the same acceleration voltage (5 kV) and resolution are used)

Carbon material		Element (%)			
		C	O	Si	S
CMP	Untreated	73.30	25.78	0.36	0.31
	Treated	77.35	31.85	–	0.37
CNF	Untreated	70.19	28.19	0.19	0.51
	Treated	62.82	35.70	0.17	0.40

untreated and treated CMPs and CNFs (introduced as DMF suspensions) in terms of oxygen percentage. It can be observed an increase of around 6% of oxygen in the treated CMPs while in the case of CNFs the oxygen percentage was increased a little bit more (8.75%). The formation of oxygen-containing surface groups occurs at defect sites on the carbon nanofibers. We could not evaluate significant changes regarding the presence of other elements (Si, S, Al, etc.).

3.2. Electrochemical characterization of CMs films on GC and Au electrode surfaces

The electrochemical properties of the CMs were examined using cyclic voltammetry (CV). The fast and reliable detection of NADH at low potential is particularly important.

The electrochemical signal transduction ability of CMs and their possible application in biosensor systems was evaluated by measuring the response of GC and Au electrode surfaces modified with treated and untreated CMPs and CNFs films.

Fig. 4 shows the cyclic voltammograms of bare Au electrode (upper CVs) and Au electrodes modified with untreated (A) and treated (B) CNFs. The same CVs for the Au electrodes modified with untreated (C) and treated (D) CMPs are also shown. A pH 7.0 PBS containing 2.5 mM NADH, a scan rate of 100 mV s^{-1} during a potential range of -0.6 to 1.2 V have been used in all cases. The CNF and CMP modified Au electrodes (CNF/Au and CMP/Au) showed two redox waves. Firstly, the NADH oxidation peaks: $+0.349 \text{ V}$ (untreated CNF); $+0.520 \text{ V}$ (treated CNF); $+0.517 \text{ V}$ (untreated CMPs) and at $+0.505 \text{ V}$ (treated CMPs). Secondly, the reduction peaks (at around 0.4 V) possibly ascribed to gold reduction appearing as a result of uncovered Au surfaces that could also be observed in the SEM images shown previously (Fig. 1).

On the other hand in the case of Au electrodes (Fig. 4) the shifts of the NADH oxidation potentials vary according to each CMs. In the case of modified Au electrode the peak of the NADH oxidation current corresponding to untreated CNF compared to the bare electrode represents a shift of around $\Delta E = +0.354 \text{ V}$. This peak represents also the highest current value comparing to the treated CNF and treated or untreated CMPs. For the Au electrodes modified with CMPs the highest oxidation potential observed ($21.04 \mu\text{A}$) corresponds to the treated CMPs. This peak (at $+0.505 \text{ V}$) represents a shift of $\Delta E = +0.198 \text{ V}$ with respect to bare Au electrode.

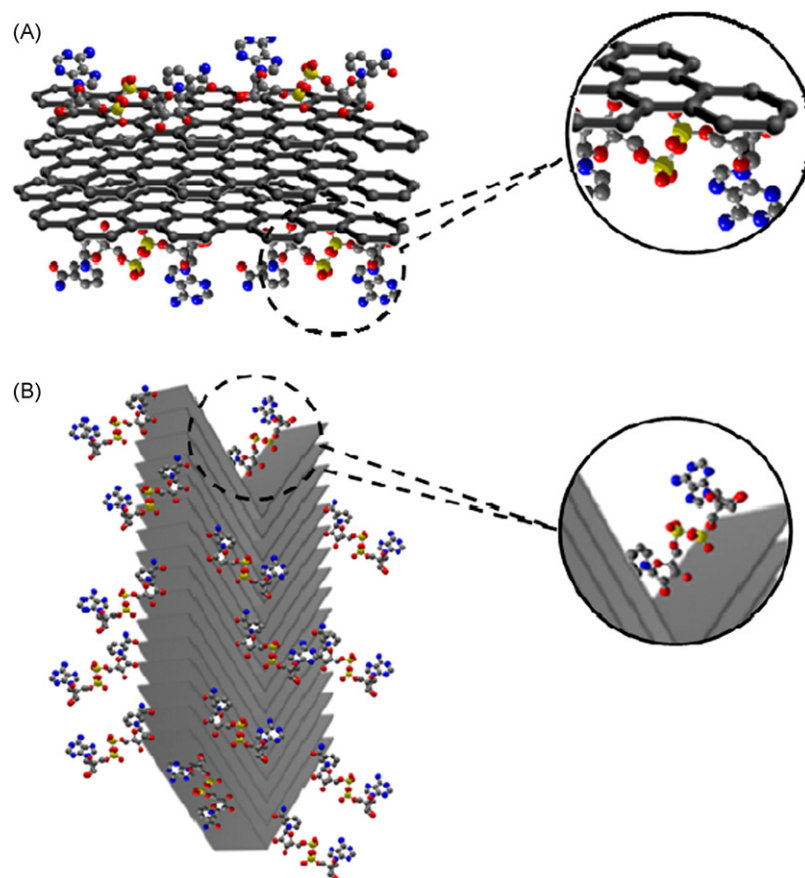


Fig. 3. Schematic representation (not in scale) of the possible interactions of the NADH molecules with: (A) graphene layers for the CMPs and (B) graphene layers for CNFs.

Fig. 5 shows the cyclic voltammograms of bare GC electrode (upper part) for a 2.5 mM NADH including the response in PBS buffer only (pH 7.0). The CVs for the GC electrodes modified with untreated (A) and treated (B) CNF are shown. The same CVs for GC electrodes modified with untreated (C) and treated (D) CMPs are also presented for the same conditions as presented for the modified Au electrodes (Fig. 4). All the CVs show clearly the NADH oxidation peaks comparing to PBS buffer. The peak currents for NADH oxidation are of a similar magnitude for each CM independently of being treated or not.

By comparing Fig. 5 (GC electrode) with Fig. 4 (Au electrode) it can be concluded that the GC electrodes utilized as substrate for deposition of carbon material films display a sig-

nificantly different responses compared to the gold electrodes (Fig. 4). The sensitivity toward NADH oxidation, in the case of electrodes modified with CMPs is higher with GC electrode (Fig. 5C and D) than with Au electrode (Fig. 4C and D) (note the differences in scale). The obtained results can be supported also by the images of Fig. 1 where better films onto GC electrode surfaces compared to those onto Au electrodes can be appreciated. Higher electrochemical responses, in both electrodes (GC and Au), have been observed for modifications using CNFs. The peak current ($84.69 \mu\text{A}$ at $+0.352 \text{ V}$) for the NADH oxidation for GC modified with untreated CNF shifted with a $\Delta E = +0.393 \text{ V}$ with respect to bare GC electrode. Lower sensitivity ($18.21 \mu\text{A}$) is observed for the Au electrode modified with untreated CMPs.

Table 2
Summary of responses toward a solution of NADH 2.5 mM at 0.1 M phosphate buffer pH 7 obtained by two different electrode transducers (GC and Au) modified with carbon nanofibers (CNFs) + dimethylformamide (DMF) and graphite + DMF

Modifier	Transducer	Glassy carbon (GC)			Gold (Au)		
		I_p (μA)	E_o (V)	ΔE (V)	I_p (μA)	E_o (V)	ΔE (V)
Graphite	Untreated	40.91	0.538	0.207	18.21	0.517	0.186
	Treated	52.42	0.535	0.210	21.04	0.505	0.198
Carbon nanofibers	Untreated	84.69	0.352	0.393	83.32	0.349	0.353
	Treated	71.28	0.505	0.240	25.58	0.520	0.183

I_p : the peak of the NADH oxidation current; E_o : NADH oxidation potential; ΔE : shift of the oxidation potentials between bare and modified electrodes.

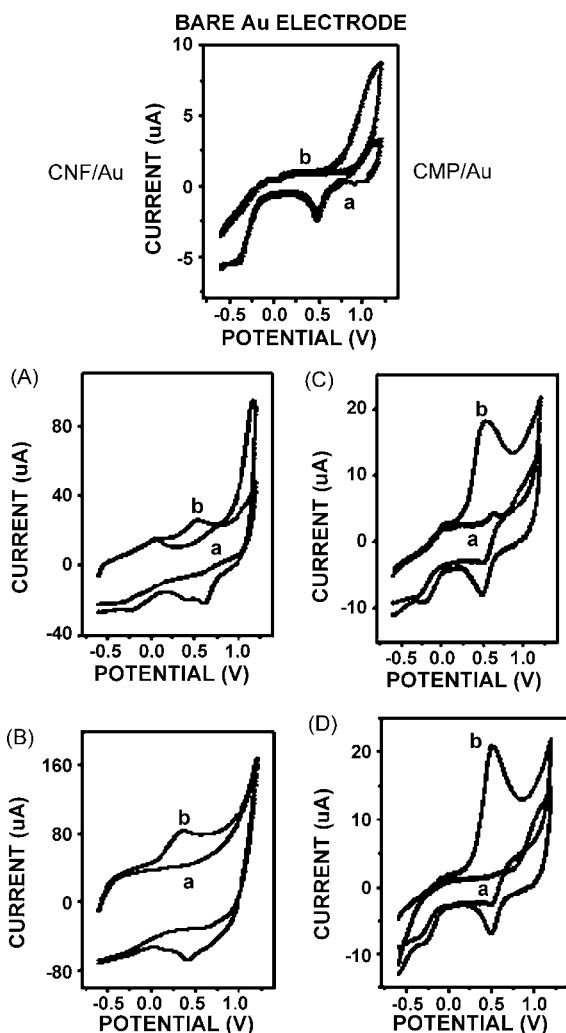


Fig. 4. Cyclic voltammograms (CV) for an 0.1 M phosphate buffer pH 7 (a) using: a bare Au electrode (upper CVs) and Au electrodes modified with untreated CNFs (A), treated CNFs (B), untreated CMPs (C), and treated (D) CMPs. The CV responses toward a 2.5 mM NADH solution (b) (at the same pH) are also shown using the same electrodes. The scan rate was 100 mV/s, potential range: -0.6 to 1.2 V. The composition of the modifying solution for all the cases was: 0.7 mg carbon material and 700 μ L DMF, respectively. Other experimental details as explained in the text.

Comparing both electrodes used (Au and GC) the sensitivity based on the current observed, increases from untreated to treated CMPs and from treated to untreated CNFs (Figs. 4 and 5).

From the obtained results, it can be concluded that the modifications with CNF brings to the electrodes higher sensitivity to the oxidation of NADH and therefore the electrochemical properties are more favourable comparing to modifications with CMPs.

Table 2 summarizes all the values (potential and currents) discussed previously for each electrode and the corresponding modifications. The shown differences can be attributed to the different structural forms of the carbon materials used. Carbon nanofibers (see Fig. 3) provide a very high-surface area and, consequently, a very high number of binding sites for NADH. This high density of binding sites may increase sensi-

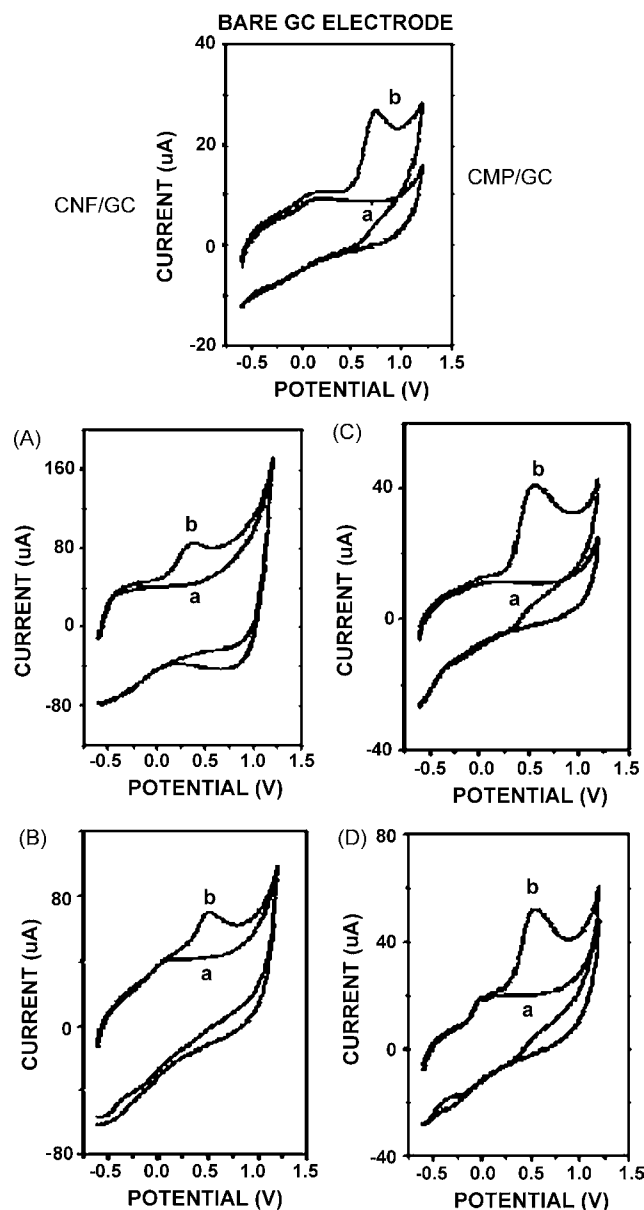


Fig. 5. Cyclic voltammograms (CV) for an 0.1 M phosphate buffer pH 7 (a) using: a bare GC electrode (upper CVs) and GC electrodes modified with untreated CNFs (A), treated CNFs (B), untreated CMPs (C), and treated (D) CMPs. The CV responses toward a 2.5 mM NADH solution (b) (at the same pH) are also shown using the same electrodes. The scan rate was 100 mV/s, potential range: -0.6 to 1.2 V. The composition of the modifying solution for all the cases was: 0.7 mg carbon material and 700 μ L DMF, respectively. Other experimental details as explained in the text.

tivity in the same manner as in porous materials [38]. Generally, electron transfer may be more facile at samples containing a higher proportion of binding sites, producing a faster electron transfer.

The principal reasons for different “electrocatalytic” properties of carbon materials on different substrates toward different analytes are still unclear and are under investigation. A possible explanation of such an effect is the different contact resistances of carbon materials (CMPs and CNFs) with the GC and Au electrode surfaces [15].

4. Conclusions

The electrochemical response for NADH oxidation is significantly different for each carbon material used as modifier as well as between GC and Au electrodes examined as substrates onto which the modifications have been performed. The results obtained show that the better substrate is the GC electrode, and the better modification is the untreated CNF film showing a peak current of $84.69 \mu\text{A}$ at $+0.352 \text{ V}$ with a shift of the oxidation potential of $+0.393 \text{ V}$ respect to bare GC electrode. The lower sensitivity is observed for the Au electrodes modified with untreated CMPs giving a peak height of $18.21 \mu\text{A}$ at $+0.517 \text{ V}$ with a shift of the oxidation potential of $+0.186 \text{ V}$ respect to bare Au electrode.

CNFs modifications show in most of the cases (except treated CNF at Au electrode) lower overpotentials and higher oxidation currents for the detection of NADH comparing to modifications performed with CMPs. The shown ability of untreated CNF to promote the electron transfer between NADH and the electrode surface suggest an attractive electrocatalytic nanomaterial for development of new amperometric biosensors. In addition to the attractive electrocatalytic properties the solubility of CNF in DMF better facilitates its manipulation for future biosensing applications.

Congratulations



A. Merkoçi

It was really a great pleasure for me (one of the authors of this article) to work during the year 2002 as scientific manager of Dr. Wang's Labchip laboratory in Las Cruces, NM. I found there a very motivated and hard working team led by a great scientist and person.

Thanks Dr. Wang for the opportunity to work with you!

I wish you all the best for this anniversary.

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References

- [1] Ü.A. Kirgoz, S. Timur, D. Odaci, B. Pérez, S. Alegret, A. Merkoçi, *Electroanalysis* 19 (2007) 893–898.
- [2] S. Alegret, A. Merkoçi, M.I. Pividori, M. del Valle, Chapter Encyclopedia of Sensors, vol. 3, in: A.G. Craig, C.D. Elizabeth, V.P. Michael (Eds.), The Pennsylvania State University, University Park, USA. Forwarded by Prof. R.A. Marcus, Nobel Prize Laureate, 2006, pp. 23–44, <http://www.aspbs.com/eos/>.

- [3] B. Pérez, M. Pumera, M. del Valle, A. Merkoçi, S. Alegret, J. Nanosci. Nanotechnol. 5 (2005) 1694–1698.
- [4] S. Alegret, A. Merkoçi, S. Alegret (Eds.), *Integrated Analytical Systems*, Elsevier, Amsterdam, 2003, pp. 377–412, ISBN: 0-444-51037-0 (NL).
- [5] A. Merkoçi, M. Pumera, X. Llopis, B. Pérez, M. del Valle, S. Alegret, *Trends Anal. Chem.* 24 (2005) 826–838.
- [6] K.-P.S. Dancil, D.P. Greiner, M.J. Sailor, *J. Am. Chem. Soc.* 121 (1999) 7925–7930.
- [7] J.M. Buriak, *Chem. Commun.* (1999) 1051–1060.
- [8] L.A. DeLouise, B.L. Miller, *Anal. Chem.* 76 (23) (2004) 6915–6920.
- [9] J. Wang, Q. Chen, C.L. Renschler, C. White, *Anal. Chem.* 66 (1994) 1988–1992.
- [10] C. Lei, Y. Shin, J. Liu, E. Ackerman, *J. Am. Chem. Soc.* 124 (2002) 11242–11243.
- [11] M. Yemini, M. Reches, E. Gazit, J. Rishpon, *Anal. Chem.* 77 (2005) 5155–5159.
- [12] S.S. Wong, E. Joselevich, A.T. Wooley, C.L. Cheung, C.M. Leiber, *Nature* 394 (1998) 52–55.
- [13] F. Valentini, A. Salis, A. Curulli, G. Palleschi, *Anal. Chem.* 76 (2004) 3244–3248.
- [14] A. Curulli, E. Valentini, G. Padeletti, A. Viticoli, D. Caschera, G. Palleschi, *Sens. Actuator B: Chem.* 111 (2005) 441–449.
- [15] M. Pumera, A. Merkoçi, S. Alegret, *Electrophoresis* 28 (2007) 1274–1280.
- [16] M. Musameh, J. Wang, A. Merkoçi, Y.H. Lin, *Electrochem. Commun.* 4 (2002) 743–746.
- [17] B. Perez, M. Pumera, M. del Valle, A. Merkoçi, S. Alegret, J. Nanosci. Nanotechnol. 5 (2005) 1694–1698.
- [18] S. Hrapovic, Y. Lui, K.B. Male, J.H. Luong, *Anal. Chem.* 76 (2004) 1083–1088.
- [19] C. Park, E.S. Engel, A. Crowe, T.R. Gilbert, N.M. Rodriguez, *Langmuir* 1 (2000) 8050–8056.
- [20] S.-U. Kim, K.-H. Lee, *Chem. Phys. Lett.* 400 (2004) 253–257.
- [21] S.H. Yoon, S. Lim, Y. Song, Y. Ota, W.M. Qiao, A. Tanaka, I. Mochida, *Carbon* 42 (2004) 1723–1729.
- [22] M.K. van der Lee, A. Jos van Dillen, J.H. Bitter, K.P. de Jong, *J. Am. Chem. Soc.* 127 (2005) 13573–13582.
- [23] H. Cui, S.V. Kalinin, X. Yang, D.H. Lowndes, *Nano Lett.* 4 (2004) 2157–2161.
- [24] H. Hacker, E. Wallnofer, W. Baumgartner, T. Schaffer, J.O. Besenhard, H. Schrottner, M. Schmied, *Electrochem. Commun.* 7 (2005) 377–382.
- [25] N.M. Rodriguez, M.-S. Kim, R.T.K. Baker, *J. Phys. Chem.* 98 (1994) 13108–13111.
- [26] N.M. Rodriguez, A. Chambers, R.T.K. Baker, *Langmuir* 11 (1995) 3862–3866.
- [27] C.A. Bessel, K. Laubernds, N.M. Rodriguez, R.T.K. Baker, *J. Phys. Chem. B* 105 (2001) 1115–1118.
- [28] P. Serp, M. Corrias, P. Kalck, *Appl. Catal.* 253 (2003) 337–358.
- [29] M. Endo, Y.A. Kim, T. Hayashi, Y. Fukai, K. Oshida, M. Terrones, T. Yanagisawa, S. Higaki, M.S. Dresselhaus, *Appl. Phys. Lett.* 80 (2002) 1267–1269.
- [30] M.J. Ledoux, R. Vieira, C. Pham-Huu, N. Keller, *J. Catal.* 216 (2003) 333–342.
- [31] G.N. Patrick, Doctoral Thesis: The Use of Bipolar Electrochemistry in Nanoscience: Contact Free Methods for the Site Selective Modification of Nanostructured Carbon materials; Development of bipolar electrodeposition as a tool for nanotechnology: Part 1 from Platelets to Nanofibers (Chapter 3).
- [32] L. Wu, X. Zhang, H. Ju, *Anal. Chem.* 79 (2007) 453–458.
- [33] C.E. Banks, R.G. Compton, *Analyst* 130 (2005) 1232–1239.
- [34] L. Li, C.M. Lukehart, *Chem. Mater.* 18 (2006) 94–99.
- [35] M. Pumera, A. Merkoçi, S. Alegret, *Sens. Actuator B* 113 (2006) 617–622.
- [36] M.S. Dresselhaus, G. Dresselhaus, *Top. Appl. Phys.* 80 (2001) 11–28.
- [37] T.G. Ros, A.J. van Dillen, J.W. Geus, D.C. Koningsberger, *Chem. Eur. J.* 8 (2002) 1151–1162.
- [38] S.E. Baker, K.-Y. Tse, E. Hindin, B.M. Nichols, T.L. Clare, R.J. Hamers, *Chem. Mater.* 17 (2005) 4971–4978.

Review

Contactless conductivity detection for microfluidics: Designs and applications

Martin Pumera^{*}*ICYS, National Institute for Materials Science, 1-1 Namiki, Tsukuba 305-0044, Japan*

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Abstract

Different methods for construction of contactless conductivity detectors (CCD) for microchip electrophoresis device are described in this review. This includes three main schemes of CCD for microchips, such as (i) the detection electrodes are placed along the microchannel from outside of the microchip and they are insulated from the channel by the cover lid of microchip device; (ii) the electrodes are placed across of the microchannel in the same plane and they are insulated by thin separation channel walls and (iii) electrodes are buried in widened part of microchannel and they are insulated from solution by ultrathin layer of silicon carbide. Specific issues related to the CCD on microfluidics are discussed, such as an influence of shape and magnitude of ac voltage and placement of electrodes and their insulation. Various applications for security, pharmacological, bioassays and food analysis purposes are described.

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Keywords: Contactless conductivity detection; Microfluidics; Lab-on-a-chip; Microchip electrophoresis; Review

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1. Introduction

Microfluidic systems (lab-on-a-chip) can dramatically change the speed and scale at which chemical and biological analyses are performed [1–4]. Such miniaturized systems rep-

^{*} Fax: +81 29 860 4706.E-mail address: PUMERA.Martin@nims.go.jp.

resent the ability to shrink conventional “benchtop” analytical systems with major advantages of speed, integration, cost, portability and sample/reagent consumption. Wide range of detection techniques was employed in microfluidic devices, such as laser induced fluorescence, mass-spectroscopy and electrochemistry. Even though there was substantial progress in miniaturization of laser induced fluorescence and mass-spectrometry detection systems [5–8], these devices are expensive, energy intense and barely portable. Electrochemical detection [9–11], especially conductivity detection [11], offers great promise for microfluidic systems, with features that include high sensitivity, intrinsic miniaturization capability, low-power requirements, compatibility with advanced microfabrication technologies and low cost.

Conductivity detection is a simple and universal detection technique for electrophoretic systems. Such detection involves measurement of the conductance between two or four electrodes through which an alternating current is passed and allows convenient detection of ionic species, which are often not readily detected by other techniques, down to the nanomolar level. Conductivity detection can be accomplished either by a direct contact of the run buffer and the sensing electrodes or by a contactless mode in which the electrodes do not contact the solution. The contactless detection route has several advantages over the contact mode, including the absence of problems (such as passivation of electrode surface, bubble formation as a result of evolution of gas due to placement of nonisolated detection electrode in high separation voltage field, the need for complicated cell geometry modification etc.) associated with the electrode-solution contact, effective isolation from high separation voltages, a simplified construction and alignment of the detector and the use of narrow micro/nano-channels [11,12].

Contactless conductivity detection (CCD) was popular as detection technique for isotachopheresis in 1980s [13,14]. It took almost two decades until rather complicated four electrode contactless conductivity detector set-up used typically in isotachopheresis for ‘across-the-capillary’ measurements was simplified to two electrode set-up for ‘along-the-capillary’ measurements (which shows favorable sensitivity for capillary electrophoresis with small capillaries due to the fact that the detection cell has length approximately equal to the gap between the electrodes [15], typically 1–2 mm) and employed for capillary electrophoresis (CE) independently by da Silva and do Lago [16] and Zemmann et al. [17] in 1998. Since then the number of publications on capillary electrophoresis with CCD exploded, mainly because do Lago published the detailed electronic scheme of the contactless conductivity detector [16]. CE/CCD showed very wide application range for detection of ionic compounds, such small inorganic ions [18], fatty acids [19], drugs [20] and immunomolecules [21]; also it proved that it can be used to detect neutral compounds such alcohols [22] or carbohydrates [23] (details on application of CCD for CE can be found in an excellent reviews, see references [24–26]).

First reports on integration of CCD to the microfluidic format emerged in years 2001 and 2002. The aim of this review is to cover the time span of the last 7 years (2001–2007) while focusing on the three main schemes of CCD implementation in microfluidics: (i) CCD electrodes are placed along the

microchannel from outside of the microchip and they are insulated from the channel by thickness of the cover microchip lid (hundreds of micrometers); (ii) the electrodes are placed across of the microchannel in the same plane and they are insulated by thin (tens of μm) layer of chip material and (iii) electrodes are buried in widened part of microchannel and they are insulated from solution by ultrathin (tens of nm) layer of silicon carbide.

2. Contactless conductivity detection for microfluidics: Designs

CCD for conventional CE used tubular electrodes placed cylindrically around the outer polyimide coating of the fused-silica capillary. Transferring such configuration to the lab-on-chip format seemed practically impossible. Therefore, Baltussen and coworkers [27] and Verpoorte and coworkers [28] chose rather complicated way of burying four electrodes in the microchip and placing them across the channel. Such configurations resemble to a certain extent CCD for isotachopheresis and are accessible only to the laboratories with advanced microfabrication capabilities. Furthermore these ‘across-the-microchannel’ designs [27,28] suffer from the same problem as CCD for isotachopheresis—strong dependency of sensitivity on the channel diameter. Solution of the problem had been overlooked until 2001 when Opekar’s group showed that CCD electrodes for CE does not have to be tubular, but they can be constructed as semi-tubular without noticeable influence on CCD performance [29]. From this point it was logical step into construction of the planar electrodes on top of the microchip lid along the channel by Wang and coworkers [30].

2.1. CCD electrodes placed outside on the microchip lid along the microchannel

This design was introduced by Wang’s group in 2002 and it has been widely adopted by other groups since then because of its simplicity and sensitivity [30]. This detection scheme for microfluidics, shown in Fig. 1, is derived from well-proved CCD detection scheme for conventional capillary electrophoresis and takes advantage from it. The longitudinal electrode alignment does not require modification of microchip. This simple contactless conductivity microchip detector is based on placing two planar sensing film electrodes on the outer side of a PMMA microchip and measuring the impedance of the solution in the microchannel. Such CCD design obviates cumbersome efforts associated with other microfluidics/CCD schemes (see Sections 2.2 and 2.3). The supposed problem of lower sensitivity which could arise from the fact that the sensitivity of CCD detector decreases with the thickness of insulation layer between the electrode and the solution [31] (in case of lab-on-chip it is the thickness of microchip lid; however, the cell size is correlated with the detection limits *indirectly*—in CCD, a background signal is always present due to the electrophoresis buffer, and the relating cell size to detection limits is not straightforward. The background signal determines to a large extent the noise level, and is also reduced for smaller cells. See additional discussion in Section 2.2 and in reference [15]) is compensated by fact that the

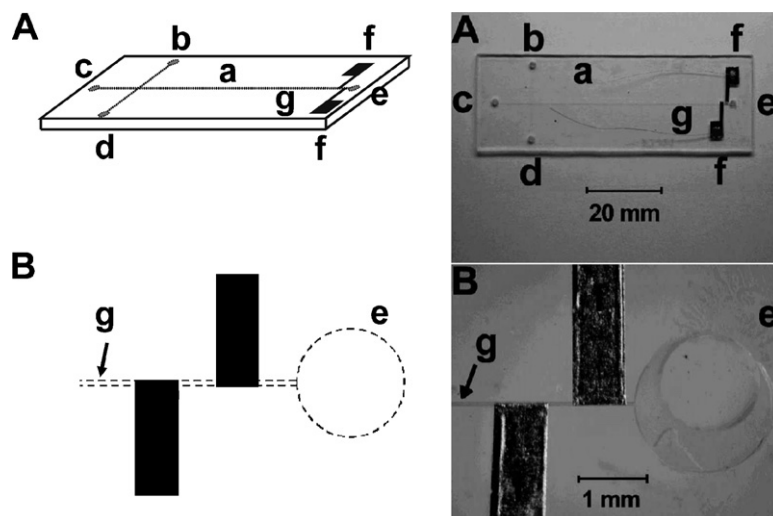


Fig. 1. Microchip electrophoretic system with contactless conductivity detection (“on top of lid” design); left part scheme, right part photograph; (A) (a) PMMA microfluidic device, (b) sample reservoir, (c) run buffer reservoir, (d) unused reservoir, (e) outlet reservoir, (f) sensing electrodes and (g) separation channel. (B) Enlarged view of the detector. Adapted from [30,55] with permission.

distance (gap) between the electrodes is 0.5–2 mm, and therefore, the detection cell volume was much higher than in other designs [27,28]. Consequently, the detection limits in first works [27,28,30] were similar to each other, in micromolar levels.

Since introduction of longitudinal CCD placement by Wang and coworkers [30] several important technical developments took place, significantly improving the sensitivity of this design, expanding its applicability and elucidating many details of this scheme. It was found that the response of the contactless conductivity detector is strongly dependent upon the waveform of the applied voltage [32]. The sine wave proved itself to offer the most favorable signal-to-noise characteristics. The square and triangle waveforms result in higher signals than the sine waveform but in connection with a substantially larger noise level [32]. Hauser’s group demonstrated that in this longitudinal electrode alignment approach the sensitivity can be greatly enhanced (by factor of 10-times when compared to the original microfluidics/CCD article by Wang and coworkers [30]) using an ac voltage with elevated peak-to-peak amplitude of several hundred volts [31]. The original work [30] placed electrodes in an “antiparallel” orientation and presumed that it minimizes the stray capacitance between them. This topic in greater detail treated later by Kubáň and Hauser [33] and indeed, the original presumption [30] was proved to be correct. Theoretical description relevant to this design can be found in references [34,35], written by do Lago and coworkers.

Advances were also made in detector set-up. Wang and coworkers originally glued a pair of planar electrodes on a polymeric chip [30]. However, in principle, the electrodes do not have to be attached to the top lid of microchip but they can be placed on chip holder or other support, as was shown independently by Wang et al. [36] and Hauser and coworkers [37] groups. This allows the use of bare chips, which can be as easily mass produced as the ones employed for fluorescence detection, which is a considerable simplification in the application of electrochemical detection for lab-on-a-chip devices [37]. Furthermore, such electrode placement can be movable, which

enhances the optimization process of microfluidics/contactless conductivity detection system via “sliding” the electrode holder [36]. The movable microchip detection system offers distinct improvements compared to common fixed-location conductivity detectors. For example, placing the detector at different locations along the microchannel offers useful insights into the separation process. Three-dimensional plots of resolution/channel length/separation voltage can be used for optimizing the separation process and selecting the analysis time. By moving the detector to a shorter effective separation length, after eluting fast-migrating ions, shorter analysis times can be achieved through faster detection of late-eluting analytes (for examples, see Fig. 2) [36].

The CCD detection system are typically used in conjunction with PMMA chips due to the simplicity of fabrication PMMA chips with thin cover lid [38]; however, different materials, such glass [21,39–41] or PDMS [42] were also used. This set-up is nowadays so well established that it is routinely used as common detection technique for testing new microchips by several laboratories around the world [43–47]. Applications of this detection scheme are discussed in the Section 3 of this review.

2.2. Electrodes are placed across of the microchannel in the same plane

Lichtenberg et al. [28,48] developed CCD with the detector electrodes set-up across the microchannel. Metal electrodes were structured in recesses in plane with the microchannel network after glass etching. The electrodes were located directly across from one another, so that the ac measurement signal was applied perpendicular to the direction of flow to obtain a good spatial confinement of the detection volume. A thin glass wall of 10–15 μm separated the electrodes from the separation microchannel to achieve the electrical insulation necessary for contactless operation (see Fig. 3). This approach had the advantage that the insulating barriers could be much thinner than for the approach described in Section 2.1 which could, in principle,

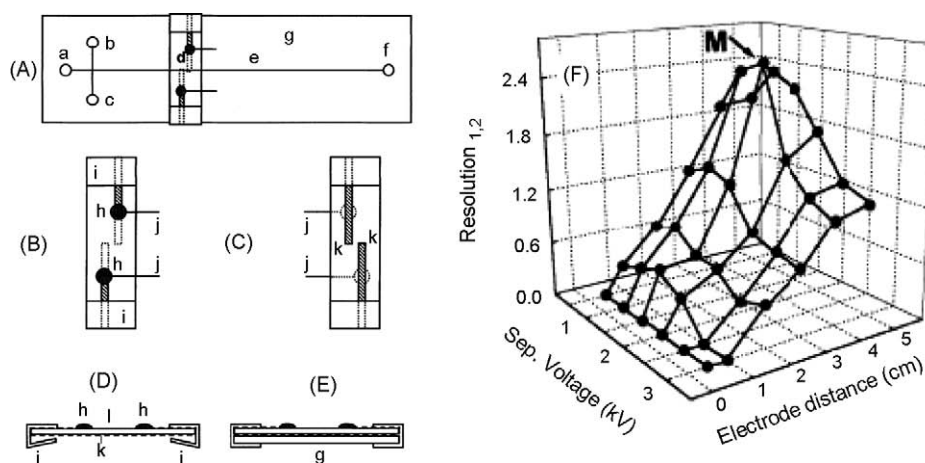


Fig. 2. Schematic diagrams of the microchip electrophoretic system with the movable CCD (A) along with a detailed design of the movable electrode system: top (B) and bottom (C) views, as well as cross-sectional views without (D) and with (E) the PMMA separation chip. (a) Run buffer reservoir, (b) sample reservoir, (c) unused reservoir, (d) movable electrodes, (e) separation channel, (f) sample waste reservoir, (g) PMMA chip, (h) conductive silver epoxy, (i) PVC clamps, (j) copper wires, (k) aluminum foil electrodes, and (l) Plexiglas plate. (F) Three-dimensional plot of resolution/channel length/separation voltage for methylammonium and ammonium. Such 3-D profile offers detailed information on the efficiency of the separation process along the channel length. The most favorable resolution of the cations in this example is represented by a wave maximum (M). Adapted from [36] with permission.

lead to higher sensitivity (due to the decrease of noise originating by stray capacitance, as discussed by Opekar and coworkers [29] and as demonstrated by Tanyanyiwa and Hauser in reference [31]). However, since the sensing in this set-up is carried out across the microchannel (typical width of channel is $50\ \mu\text{m}$), the detection volume is much lower than in Wang's design, and therefore, the limit of detection in Lichtenberg et al.'s work

[28] was found to be comparable to Wang and coworker's work [30]. The fabrication of such device did require clean room processing and maybe for this reason this work was not followed by many researchers. Recently, Lee et al. [49] demonstrated that sensitivity of Lichtenberg's design could be improved by using semicircular electrodes instead of planar electrodes (see Fig. 4). Applications of this detection scheme are discussed in the Section 3 of this review.

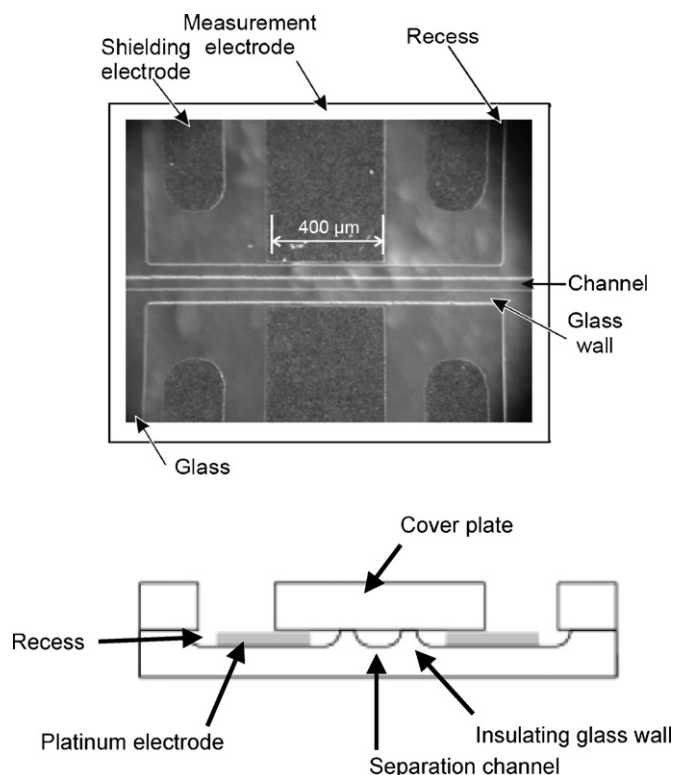


Fig. 3. Sketch of the contactless conductivity detector ("in plane" design) in a cross-section perpendicular to the flow direction in the separation channel. The two platinum electrodes are separated from the electrolyte (and high separation field) in the channel by two glass walls. Reprinted with permission from [28].

2.3. Electrodes are buried in widened part of microchannel

Guijt et al. published first article on contactless conductivity detector for microfluidics [27] (2001). To minimize problems associated with contact conductivity detection they covered the in-channel sensing electrodes with a dielectric (30 nm SiC) layer (see Fig. 5). Unfortunately, silicon carbide coating stability was limited to low separation fields (maximum $50\ \text{V cm}^{-1}$) and for this reason to long analysis times are required. Furthermore, such design requires a cumbersome design and fabrication effort, along with a widened channel which causes additional zone broadening. Following improved designs presented by the same authors were able to withstand separation fields up to $167\ \text{V cm}^{-1}$ [50] and $250\ \text{V cm}^{-1}$ (1500 V for 6 cm long microchip) [51,52] (note that design described in Section 2.1 is able to efficiently insulate high separation voltages at least $3\times$ higher). Presumably due to cumbersome design and fabrication effort no other groups adapted this design.

3. Contactless conductivity detection for microfluidics: Applications

3.1. Security

3.1.1. Explosives

A contactless conductivity detection is an ideal tool for detecting improvised explosives consisting from nitrate and per-

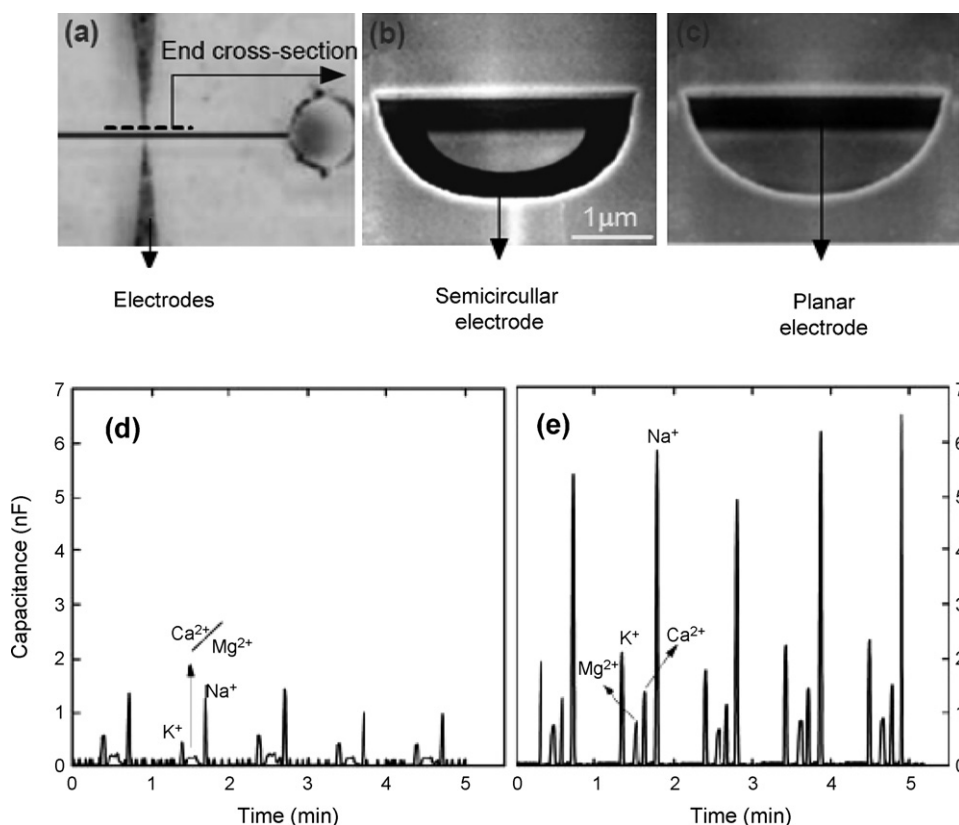


Fig. 4. Improved “in plane” design using semicircular electrodes. Photographs of semicircular and planar Au electrodes in the bonded chips: (a) planar view, (b) cross-sectional view of the channel with a semicircular Au electrode and (c) cross-sectional view of the channel with a planar electrode. Also shown is the response of the detectors for separation of anions (K⁺, Mg²⁺, Ca²⁺ and Na⁺) in CCD using (d) planar contactless electrodes and (e) semicircular detection electrodes for sample sports drink. Reprinted with permission from [49].

chlorate salts (ammonium, potassium or sodium) [53] since these are small ions with high mobility. A miniaturized analytical system for separating and detecting inorganic explosive residues, based on the coupling of a microchip electrophoresis with a contactless conductivity detector was described by Wang et al. [54,55]. The low electroosmotic flow (EOF) of the PMMA chip material facilitated the rapid switching between analyses of cations and anions using the same microchannel and run buffer (and without an EOF modifier), and therefore, resulted in rapid (<1 min) measurement of seven explosive-related cations and anions (ammonium, methyl ammonium, potassium, sodium, perchlorate, chlorate, nitrate). The separation of improvised explosive-related cations was further optimized with movable CCD (see Fig. 2F) [36]. Movable CCD was also employed in interesting novel dual-injection PMMA microchip electrophoretic system for simultaneous measurements of explosive-related anions and cations using a single channel [56]. It consisted of two sample reservoirs, on both sides of a common separation channel. Anions and cations could be simultaneously electrokinetically injected into both ends of the separation channel. Due to low electroosmotic flow in polymer channel, the cations and anions migrated in opposite directions and were separated from each other and detected using a movable CDD positioned around the center of the separation channel. In order to detect improvised (ionic) explosives

simultaneously with the industrial/military explosives (based on 2,4,6-trinitrotoluene related compounds), the CCD detection system was combined with amperometric detection system in one microchip device [39]. The behavior of the dual detector was comparable with those of the individual detectors, no cross-talk between contactless conductivity and amperometric detection unit was observed.

3.1.2. Nerve agents

The degradation products of organophosphate nerve agents (breakdown products of Sarin, Soman and VX nerve agents) are small alkyl methylphosphonic acid anions which are difficult to detect by conventional detection techniques [57]. However, similar to ionic explosives, these ions can be easily detected by contactless conductivity detector. Wang et al. [32] developed the microfluidic system relying on an efficient chip-based separation of alkyl methylphosphonic acids followed by their sensitive contactless conductivity detection. Potential for the analysis of the nerve agent residues spiked in river water samples was also demonstrated. Movable contactless conductivity detector described previously was also applied to the analysis of nerve agents degradation products [36,56]. Interesting approach to detect organophosphate nerve agents (such paraxon and methyl parathion) developed by Wang et al. was based on an on-chip enzymatic reaction of organophosphate

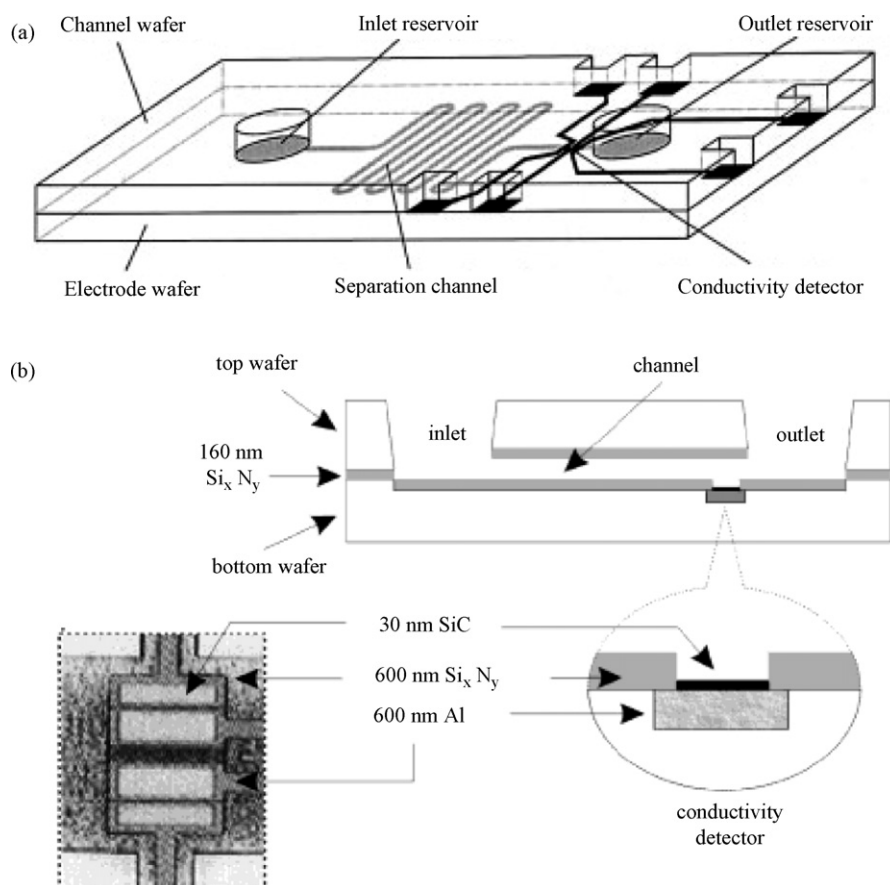


Fig. 5. Drawing of a microchip electrophoresis separation with four electrode contactless conductivity detector in "buried electrode" design; (a) 3-D view and (b) side schematic view. The photograph and the schematic inset show a closer view of the detector. Reprinted with permission from [50] and [52].

nerve agents in pre-column chamber with organophosphorus hydrolase (OPH) and their contactless conductivity detection [58].

3.2. Biomolecules

Significant advantage of CCD is its capability to detect underivatized biomolecules of different sizes and biological functions. Several articles, published mostly by Hauser's group, demonstrated the detection of underivatized amino acids, sugars, immunoglobulin IgG, aromatic amines and DNA fragments [41,59–61]. The analysis of sugars (which do not carry charge in neutral pH) was performed in strongly alkaline media to ionize the carbohydrate molecules [59]. The applicability spectrum of CCD on chip for biologically active compounds was further widened by Hauser to β -adrenergic blocking agents [20] and Vitamin C [62]. Label-free contactless conductivity monitoring of immunoreaction was demonstrated by Abad-Vilar et al. [21]. The detection of human immunoglobulin M (IgM) was performed using contactless conductivity detection on glass and PMMA microdevices. Also the analysis of IgG (an anti-human IgM) and the complex formed in the reaction between the two immunoreagents was achieved on microfluidic platform with CCD [21]. The reproducibility of the analytical signal was high (R.S.D. $\sim 1\%$), sensitivity and limits of detection obtained for

IgM ($3.4 \times 10^{-10} \text{ g mL}^{-1}$) are very good for label-free detection technique.

3.3. Food analysis

The food analysis is very important area of research. However, due to the complexity of the food samples, the food analysis is a challenge for microfluidic systems [63]. For this reasons, the analysis of food samples by microfluidic devices using CCD is limited to beverages. Kubáň and Hauser developed microchip CE/CCD method for analysis of inorganic ions, such as ammonium, sodium, magnesium, calcium and small organic anions, such formate, acetate etc. in vine, fruit juice and beer [64]. Similarly, Lee et al. used in-plane CCD for analysis of inorganic ions in sport drinks, mineral water and wine [49] (for example, see Fig. 4d and e).

4. Conclusion

Contactless conductivity detection proved to be very useful tool for analysis of samples in microfluidics, especially due to its inherent favorable properties, such as insulation of electrodes from solution, and label-free detection capability. Applications to biological samples are very promising and the commercialization of CCD for microfluidics can be foreseen in near future.

Further development towards further improved limits of detection and towards deeper understanding factors governing the signal response can be also forecast.

Congratulations

Dear Dr. Wang,
the time at NMSU was the most intensive and rewarding period in my personal and professional life. Happy Birthday!



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References

- [1] T. Vilkner, D. Janasek, A. Manz, *Anal. Chem.* 76 (2004) 3373.
- [2] M. Pumera, *Talanta* 66 (2005) 1048.
- [3] P.S. Dittrich, K. Tachikawa, A. Manz, *Anal. Chem.* 78 (2006) 3887.
- [4] A. Ríos, A. Escarpa, M.C. González, A.G. Crevillén, *Trends Anal. Chem.* 25 (2006) 467.
- [5] R.F. Renzi, J. Stamps, B.A. Horn, S. Ferko, V.A. VanderNoot, J.A.A. West, R. Crocker, B. Wiedenman, D. Yee, J.A. Frutetel, *Anal. Chem.* 77 (2005) 435.
- [6] B.E. Erickson, *Anal. Chem.* 76 (2004) 305A.
- [7] H. Yin, K. Killeen, R. Brennen, D. Sobek, M. Werlich, T. van de Goor, *Anal. Chem.* 77 (2005) 527.
- [8] M.-H. Fortier, E. Bonnell, P. Goodley, P. Thibault, *Anal. Chem.* 77 (2005) 1631.
- [9] W.R. Vandaveer IV, S.A. Pasas-Farmer, D.J. Fischer, C.N. Frankenfeld, S.M. Lunte, *Electrophoresis* 25 (2004) 3528.
- [10] J. Wang, *Electroanalysis* 17 (2005) 1133.
- [11] M. Pumera, A. Merkoçi, S. Alegret, *Trends Anal. Chem.* 25 (2006) 219.
- [12] K. Mayrhofer, A.J. Zemmann, E. Schnell, G.K. Bonn, *Anal. Chem.* 71 (1999) 3828.
- [13] B. Gaš, J. Vacík, *Chem. Listy* 74 (1980) 652.
- [14] B. Gaš, M. Demjanenko, J. Vacík, *J. Chromatogr.* 192 (1980) 253.
- [15] P. Tůma, F. Opekar, K. Štulík, *Electrophoresis* 23 (2002) 3718.
- [16] J.A.F. da Silva, C.L. do Lago, *Anal. Chem.* 70 (1998) 4339.
- [17] A.J. Zemmann, E. Schnell, D. Volgger, G.K. Bonn, *Anal. Chem.* 70 (1998) 563.
- [18] V. Unterholzner, M. Macka, P.R. Haddad, A. Zemmann, *Analyst* 127 (2002) 715.
- [19] M.A.L. Oliveira, C.L. do Lago, M.F.M. Tavares, J.A.F. da Silva, *Química Nova* 26 (2003) 821.
- [20] J. Tanyanyiwa, P.C. Hauser, *Electrophoresis* 25 (2004) 3010.
- [21] E.M. Abad-Villar, J. Tanyanyiwa, M.T. Fernandez-Abedul, A. Costa-García, P.C. Hauser, *Anal. Chem.* 76 (2004) 1282.
- [22] J.A.F. da Silva, C.L. do Lago, *Electrophoresis* 21 (2000) 1405.
- [23] M. Pumera, I. Jelínek, J. Jindřich, *Fresenius J. Anal. Chem.* 369 (2001) 666.
- [24] V. Sonlinová, V. Kašička, *J. Sep. Sci.* 29 (2006) 1743.
- [25] P. Kubáň, P.C. Hauser, *Electroanalysis* 16 (2004) 2009.
- [26] R.M. Guijt, C.J. Evenhuis, M. Macka, P.R. Haddad, *Electrophoresis* 25 (2004) 4032.
- [27] R.M. Guijt, E. Baltussen, G. van der Steen, H. Frank, H. Billiet, T. Schalkhammer, F. Laugere, M. Vellekoop, A. Berthold, L. Sarro, G.W.K. van Dedem, *Electrophoresis* 22 (2001) 2537.
- [28] J. Lichtenberg, N.F. de Rooij, E. Verpoorte, *Electrophoresis* 23 (2002) 3769.
- [29] P. Tůma, F. Opekar, I. Jelínek, *Electroanalysis* 13 (2001) 989.
- [30] M. Pumera, J. Wang, F. Opekar, I. Jelínek, J. Feldman, H. Lowe, S. Hardt, *Anal. Chem.* 74 (2002) 1968.
- [31] J. Tanyanyiwa, P.C. Hauser, *Anal. Chem.* 74 (2002) 6378.
- [32] J. Wang, M. Pumera, G. Collins, A. Mulchandani, *Anal. Chem.* 74 (2002) 6121.
- [33] P. Kubáň, P.C. Hauser, *Lab. Chip* 5 (2005) 407.
- [34] J.G.A. Brito-Neto, J.A. Fracassi da Silva, L. Blanes, C.L. do Lago, *Electroanalysis* 13 (2005) 1198.
- [35] J.G.A. Brito-Neto, J.A.F. da Silva, L. Blanes, C.L. do Lago, *Electroanalysis* 17 (2005) 1207.
- [36] J. Wang, G. Chen, A. Muck Jr., *Anal. Chem.* 75 (2003) 4475.
- [37] J. Tanyanyiwa, E.M. Abad-Villar, M.T. Fernández-Abedul, A. Costa-García, W. Hoffmann, A.E. Guber, D. Herrmann, A. Gerlach, N. Gottschliche, P.C. Hauser, *Analyst* 128 (2003) 1019.
- [38] J. Wang, M. Pumera, M.P. Chatrathi, A. Escarpa, R. Konrad, A. Griebel, W. Dörner, H. Lowe, *Electrophoresis* 23 (2002) 596.
- [39] J. Wang, M. Pumera, *Anal. Chem.* 74 (2002) 5919.
- [40] J. Wang, M. Pumera, *Anal. Chem.* 75 (2003) 341.
- [41] Z. Chen, Q. Li, O. Li, X. Zhou, Y. Lan, Y. Wei, J. Mo, *Talanta* 71 (2007) 1944.
- [42] S.F. Zhang, L.S. Wang, Z.G. Chen, Z. Dang, H.Q. Chen, X.R. Deng, *Chin. J. Anal. Chem.* 34 (2006) 1501.
- [43] C.L. do Lago, C.A. Neves, D. Pereira de Jesus, H.D.T. da Silva, J.G.A. Brito-Neto, J.A.F. da Silva, *Electrophoresis* 25 (2004) 3825.
- [44] A. Muck Jr., J. Wang, M. Jacobs, G. Chen, M.P. Chatrathi, V. Jurka, Z. Výborný, S.D. Spillman, G. Sridharan, M.J. Schöning, *Anal. Chem.* 76 (2004) 2290.
- [45] G. Chen, J. Li, S. Qu, D. Chen, P. Yang, *J. Chromatogr. A* 1094 (2005) 138.
- [46] S. Qu, X. Chen, D. Chen, P. Yang, G. Chen, *Electrophoresis* 27 (2006) 4910.
- [47] Y. Chen, P. Yang, J. Li, D. Chen, G. Chen, *Anal. Bioanal. Chem.* 384 (2006) 683.
- [48] J. Lichtenberg, A Microchip-based ion analysis system, Ph.D. Thesis, SAMLAB, Neuchâtel, Switzerland, 2002.
- [49] C.-Y. Lee, C.M. Chen, G.-L. Chang, C.-H. Lin, L.-M. Fu, *Electrophoresis* 27 (2006) 5043.
- [50] A. Berthold, F. Laugere, H. Schellevis, C.R. de Boer, M. Laros, R.M. Guijt, P.M. Sarro, M.J. Vellekoop, *Electrophoresis* 23 (2002) 3511.
- [51] F. Laugere, G.W. Lubking, J. Bastemeijer, M.J. Vellekoop, *Sens. Actuat. B* 83 (2002) 104.
- [52] F. Laugere, R.M. Guijt, J. Bastemeijer, G. Steen, A. Berthold, E. Baltussen, P. Sarro, G.W.K. Dedem, M. Vellekoop, A. Bossche, *Anal. Chem.* 75 (2003) 306.
- [53] M. Pumera, *Electrophoresis* 27 (2006) 244.
- [54] J. Wang, M. Pumera, G. Collins, F. Opekar, I. Jelínek, *Analyst* 127 (2002) 719.
- [55] M. Pumera, J. Wang, *JALA* 7 (2002) 47.
- [56] J. Wang, G. Chen, A. Muck Jr., G.E. Collins, *Electrophoresis* 24 (2003) 3728.
- [57] M. Pumera, *J. Chromatogr. A* 1113 (2006) 5.
- [58] J. Wang, G. Chen, A. Muck Jr., M.P. Chatrathi, A. Mulchandani, W. Chen, *Anal. Chim. Acta* 505 (2004) 183.
- [59] J. Tanyanyiwa, E.M. Abad-Villar, P.C. Hauser, *Electrophoresis* 25 (2004) 903.
- [60] X.Y. Gong, P.C. Hauser, *Electrophoresis* 27 (2006) 4375.
- [61] E.M. Abad-Villar, P. Kubáň, P.C. Hauser, *Electrophoresis* 26 (2005) 3609.
- [62] W.S. Law, P. Kubáň, J.H. Zhao, S.F.Y. Li, P.C. Hauser, *Electrophoresis* 26 (2005) 4648.
- [63] A. Escarpa, M.C. González, A.G. Crevillén, A.J. Blasco, *Electrophoresis* 28 (2007) 1002.
- [64] P. Kubáň, P.C. Hauser, *Electrophoresis* 26 (2005) 3169.

Editorial

Micro- and nanotechnology in electrochemical detection science

This special issue on Micro- and Nanotechnology in Electrochemical Detection Science is devoted to honor Prof. Joseph Wang's 60th birthday.

Prof. Joseph Wang is a great electrochemist and scientist who has published over 700 articles throughout his career. He is an extremely innovative, persistent, and hardworking person with ground-breaking ideas. To those who know his excellent work it is not surprising that he is the most cited scientist in the world in the fields of Chemistry and Engineering. He has received many awards for his research, such as the Heyrovsky Memorial Medal (1994, Czech Republic), two awards from the American Chemical Society, one for Chemical Instrumentation in 1999 and one for Electrochemistry in 2006, just to mention few. He has also received several honorary doctorates from numerous universities around the world.

To us, the most stunning characteristic of Prof. Joseph Wang is not the awards that he highly deserves, but rather his great personality. His legacy is not only found in his excellent research; it is in the great example he sets for us all, the inspiration he has been for many young scientist over the years. Even though he is on top of the Chemistry world, he has never allowed his associates to call him "Professor." He has always been "Dr. Wang" to them. Even though he is extremely busy with his many responsibilities, he always finds time to discuss ongoing research; he is always open to listening to the ideas of his students and post-docs. He is a true illustration of the fact that "you can teach people with words but educate them only by example." We look to Dr. Wang as the gold standard, we keep an eye on each of his new papers to see the emerging fields and directions in electrochemistry, we follow his example of consistency in dealing with grants, we try to write our articles as clearly and informatively as he does, and above all, we always find time for our associates and students—we try to motivate them just as we were motivated and encouraged by Dr. Wang. We consider ourselves extremely lucky that we have had the opportunity to work under his careful guidance at his laboratory.

This special issue was organized to address the topic of Dr. Wang's long-standing interest, with his former students and associates (now well-established scientists) and his long-term collaborators contributing their articles related to research they once performed at Dr. Wang's laboratory. What you have before you is a fine collection of articles on Micro- and Nanotechnology in Electrochemical Detection Science; however, please note that only a small fraction of Dr. Wang's former students, associates and colleagues contributed. If all of them were asked to do so, this special issue would take up several volumes. We really appreciate all of the excellent contributions we have received.

This special issue would not have been possible without the help of the editor-in-chief of Talanta, Prof. Garry Christian (University of Washington), and of Mrs. Reina Bolt and Mrs. Siobhan O'Brien from the Elsevier editorial office. We are greatly indebted to all of them for the support they have given this project.

To end this editorial we just want to say:

Happy 60th birthday, Dr. Wang!

We wish you many productive years. We had a wonderful time working with you. We will never forget it. Many thanks!

Managing Guest Editor
Martin Pumera*

National Institute for Materials Science, Tsukuba, Japan

Guest Co-editor
Alberto Escarpa

Department of Analytical Chemistry and Chemical Engineering, University of Alcalá, Alcalá de Henares, Madrid, Spain

* Corresponding author.

E-mail address: PUMERA.Martin@nims.go.jp (M. Pumera)

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Electrochemical detection of osmium tetroxide-labeled PCR-products by means of protective strands

Thomas Reske^a, Maren Mix^{a,b}, Hubert Bahl^b, Gerd-Uwe Flechsig^{a,*}

^a University of Rostock, Department of Chemistry, Dr. -Lorenz-Weg 1, D-18051 Rostock, Germany

^b University of Rostock, Department of Biological Sciences, A.-Einstein-Str. 3, D-18051 Rostock, Germany

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Abstract

This communication reports about how single-stranded 136 base polymerase chain reaction (PCR) products labeled with electrochemically active osmium tetroxide bipyridine can be detected voltammetrically by hybridization with probe strands immobilized on gold electrodes. These electroactive ssDNA targets have been obtained by means of Lambda Exonuclease treatment of the double-stranded PCR products followed by hybridization of the remaining single strands with short protective strands and covalent labeling with osmium tetroxide bipyridine. Square-wave voltammetric signals of these osmium labels have been obtained only upon hybridization with the immobilized probe strands. An optimal 50 °C hybridization temperature has been found with a saturation of the probe layer at 30 min hybridization time and 7.5 nmol/l target concentration. The blank capture probe layer alone did not yield any signal. Unprotected strands produced almost no interference. Such double-selective switch-on electrochemical hybridization assays hold great promise for the specific detection of PCR products.

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Keywords: PCR product; Protective strand; Osmium tetroxide bipyridine; Gold electrode; Probe SAM; DNA hybridization

1. Introduction

Nucleic acid-modified electrodes used as biosensors can detect the hybridization of two complementary DNA single strands (probe and target). This electrochemical transduction can be performed by means of molecules that are (a) covalently attached to the target, (b) interacting with the negatively charged phosphate groups or (c) intercalating into the base stack. It is also possible to make use of the intrinsic electrochemical activity of the DNA, e.g. the oxidation of guanine [1–3]. Magnetic beads [4,5] and self-assembled monolayers [6–12] are proven approaches to immobilize the probe strands. We recently reported about the application of covalently attached redox markers together with thiol-linked probe oligonucleotides on heated gold electrodes [13,14].

A few studies about the electrochemical detection of polymerase chain reaction (PCR) products have been published.

Methylene blue intercalates stronger into ssDNA (single-stranded DNA) than into dsDNA (double-stranded DNA). This has been used to detect the hybridization of PCR products with capture probes immobilized on carbon paste electrodes [15]. Upon successful hybridization, a diminished voltammetric methylene blue signal has been observed. In similar approaches, daunomycin [16,17] and Meldola's blue [18] have been used to detect PCR products electrochemically. Hybridization detection assays basing on intercalators and alike often suffer from small differences in the electrochemical signals obtained before and after target binding. Yet, this difference has to be taken as the hybridization indication.

The methods of electrochemical hybridization detection have been reviewed several times [19–23].

The complex [OsO₄(bipy)] can be utilized as an electrochemically reversible covalent DNA-label [24]. It reacts with the pyrimidine bases thymine and cytosine [25] forming a diester of osmium(VI) acid in well-known manner under oxidation of the C–C-double bond in the pyrimidine ring. Labeling of DNA-strands of any length can be easily performed in the laboratory, which is an important advantage of this approach. However,

* Corresponding author. Tel.: +49 381 4986470; fax: +49 381 4986461.
E-mail address: gerd-uwe.flechsig@uni-rostock.de (G.-U. Flechsig).

only pyrimidine bases of single strands can be labeled this way. Such Os-modified DNA strands on the other hand are unable to form double strands, i.e. hybridization with probe oligos is no longer possible. These reactions have been investigated for about 25 years by Palecek et al. They showed that osmium tetroxide complexes could be utilized as strand selective probes which allow not only discriminating between single and double-stranded DNA but also studying the structure of double strands as well as sensitive detection of nucleic acids [26–29]. Until now, the steps of Os(VIII)-labeling, probe hybridization and electrochemical detection have been conducted in solution and at separate surfaces, respectively, especially by means of magnetic beads [30–34]. Such osmium tetroxide labeling had also been applied to detect oligo(dA)_n-containing PCR products by means of thermal denaturation and magnetic separation [31,32].

Protective oligonucleotides are known from biochemical applications, where, e.g. DNA strands have to be protected against cleavage by nucleases [35]. Recently, we reported on how protective oligonucleotide strands can be utilized for labeling thymine-containing nucleic acids by means of [OsO₄(bipy)] without losing their ability for hybridization reactions with immobilized oligonucleotide probes [36,37]. We applied this technique to compare the DNA hybridization on both heated ceramic-based and rotating gold disk electrodes [38]. Fojta et al. have (independently from us) reported about the use of protective oligonucleotides for the preparation of osmium tetroxide-labeled reporter strands by means of magnetic separation [39]. Here, we demonstrate that this new method can also be applied to detect the products of polymerase chain reactions (PCR). Such amplicons are generally obtained in the form of double strands. To ease the hybridization with the immobilized probe oligonucleotides, we removed one of the product strands by nuclease cleavage.

2. Experimental

2.1. Material

2,2'-Bipyridine was delivered by Merck and osmium tetroxide as a 2% aqueous solution from Fluka. The dithiol-modified DNA probe (*F-probe*) containing a (dT)₁₅-spacer was delivered by Friz Biochem GmbH (Munich, Germany). The 136 mer *Fragment* was prepared as described below. The “Tris-buffer” contained 10 mmol/l Tris-(hydroxymethyl)-aminomethane and 0.5 mol/l sodium sulfate and was adjusted to pH 7.5 using sulfuric acid. The sequences of all DNA-strands together with their length and designations are given in Table 1. The *F-probe* oligonucleotide fits to a 27-base recognition site (underlined in Table 1) of the 136 base *Fragment*. The *F-protector* oligonucleotide contains 5 mismatches (underlined and bold in Table 1) to ease its later displacement by the immobilized *F-probe*.

2.2. PCR and Exonuclease treatment conditions

A synthetic 136-base-DNA (sequence from the genome of *Clostridium acetobutylicum* [1855609–1855744]) was used as template. The standard PCR reaction was performed with

Table 1
DNA sequences

Designation	Length	Sequence
<i>Fragment</i>	136	5'- CTTACAGGAATTGTTTTGGGACTTGCAA GGGCTTTTGGAGAGGCATTAGCAGTTCAA ATGGTAATAGGTAATACAGTAAATCAG CTCAAGGACTTTTTTCTCCTAC- TACCACTTTGACAAGCGTACTTACAATGG- 3'
<i>F-protector</i>	27	5'-CAG TGT TTG ACC TGA TTT TAC TGT CTT-3'
<i>F-probe</i>	27	5'-AAG TCC TTG AGC TGA TTT TAC TGT ATT- tttttttttttt-[Dithio] ₃ -3'

100 ng of template DNA, 0.4 μM dNTPs, 0.5 μM of the primers C.aceto F and C.aceto Rp (Table 2) and 2.5 U Sawady-Taq-DNA-Polymerase (Peglab Biotechnologie GmbH, Erlangen, Germany) in 50 μl reaction volume according to the manufacturer's instructions. While synthetic primers are normally not phosphorylated on their 5'-end (according to natural DNA), the primer C.aceto Rp was 5'-phosphorylated for later recognition by the exonuclease to allow cleavage of only this strand. The enzymatic hydrolysis of the phosphorylated strand of the DNA was performed using 15 U of Lambda Exonuclease (NEB GmbH, Frankfurt a.M., Germany), which has the highest selectivity for 5'-end phosphorylated DNA, in 1× Lambda Exonuclease buffer for 1 h at 37 °C. During this step, the phosphorylated strand was recognized by the exonuclease and (at least in theory) completely removed from the PCR product's double strand releasing a single strand. We will show in the next section that the yield of this process was not 100%. The enzyme was heat-inactivated after the reaction for 10 min at 75 °C. Best results in the voltammetric experiments were achieved when the DNA was purified after that using the NucleoSpin Extract II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with 4 volumes of NTC buffer or the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) according to the manufacturer's instructions. This purified mainly single-stranded PCR product called *Fragment* has been used in the following steps. All DNA concentrations were evaluated visually in the agarose gel (2%, w/v) using the DNA-marker (Fermentas GmbH, St. Leon-Roth, Germany) as a comparison.

2.3. Modification of *Fragment* oligonucleotide

The *Fragment* at its maximum concentration (between 25 and 250 nmol/l) and the protective strand *F-protector* (10 μmol/l) in at least ten-fold excess were mixed and left for 2 h at room temperature to allow hybridization. In one control experiment, the protective strand has not been added. A Tris-buffer solution

Table 2
Primers

	Sequence: 5'-3'	
C.aceto F	CTTACAGGAATTGTTTTGGGA	Forward primer
C.aceto Rp	p CCATTGTAAGTACGCTTGTC	Reverse primer

containing 10 mmol/l osmium tetroxide and 10 mmol/l 2,2'-bipyridine (forming $[\text{OsO}_4(\text{bipy})]$) was then added and left at room temperature for 2 h. After this period, the excess of the osmium complex was removed by dialysis against pure Tris-buffer by means of Slide-A-Lyzer MINI Dialysis Units, 3500 MWCO (Rockford, IL, USA) at 4 °C for 19 h. This solution (containing the osmium-labeled target *Fragment*) was used for the hybridization experiments.

2.4. Preparation of SAM-modified gold electrodes

The gold disk electrode (Metrohm, Switzerland) was first polished using corundum 0.3 μm and then electrochemically treated by means of 25 voltammetric cycles between -0.2 and $+1.65$ V at 100 mV/s in 0.5 M sulfuric acid. After rinsing with water, one droplet of 15 μl *F-probe* (30.3 $\mu\text{mol/l}$) solution was given onto the dried electrode and left overnight (16 h) at 10 °C in a water-saturated atmosphere allowing the formation of the probe-SAM. After this, the electrode was soaked for 1 h in 1 mmol/l aqueous mercaptohexanol solution.

2.5. Hybridization and dehybridization procedure

During the hybridization step, the rotating gold disk electrode was dipped in a beaker containing the target (*Fragment*) solution and adjusted to 1000 rpm. After this the electrode was rinsed with Tris-buffer. To regenerate the probe-SAM by dehybridization, the electrode was immersed in 50 °C water for 60 s.

2.6. Electrochemical measurements

For all electrochemical procedures, an Autolab PSTAT10 (ecochemie, Utrecht, The Netherlands) controlled by a PC with GPES 4.8 software and an electrode stand inside a *Faraday* cage together with a Ag/AgCl (3 mol/l KCl) reference electrode and a glassy carbon counter electrode (all from Metrohm AG, Herisau, Switzerland) was used. The parameters for square-wave voltammetry were 200 Hz, 40 mV amplitude. The scan was performed from -0.55 to 0.0 V. All measurements were conducted at room temperature in Tris-buffer. The peak-shaped signals were smoothed and baseline-corrected. Automatic peak search was applied.

3. Results and discussion

The PCR and Lambda Exonuclease treatment were performed as described above. For a successful electrochemical detection without disturbances of hybridization of probe and target, we chose a sequence with a low ability to form stable secondary structures. The results of PCR and Lambda Exonuclease treatment are displayed in Fig. 1. In lane 1, the molecular marker was applied, in lane 2, the product of the PCR before Lambda Exonuclease treatment and in lane 3, the PCR-product after Lambda Exonuclease treatment is shown. It can be seen that the PCR resulted in a single band, which is then partially digested after the Lambda Exonuclease treatment. The ssDNA shows a different running behavior in the gel. Such changed

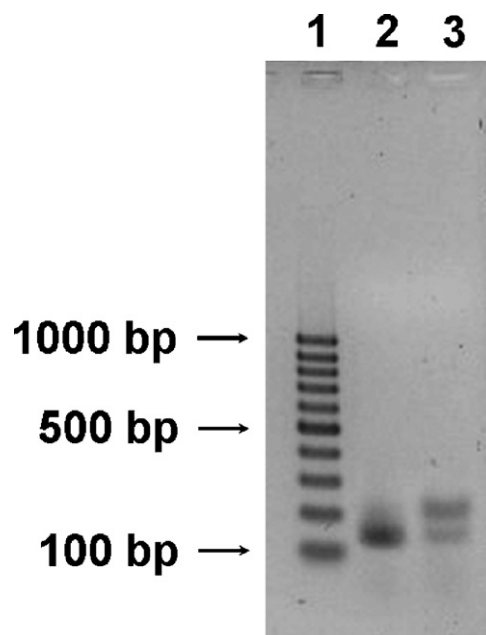


Fig. 1. Two percent (w/v) agarose gel stained with Gelred (Cambridge Bio-Science, UK): lane 1, Gene Ruler™ 100 bp DNA ladder (0.3 μg); lane 2, 2 μl PCR-product (136 bp-*Fragment*); lane 3, 5 μl PCR-product after Lambda Exonuclease treatment.

interactions with the agarose gel can be attributed to a different ternary structure of ssDNA, which is bulkier compared to dsDNA, which is more stretched. A major fraction of the PCR product has been digested to the single-stranded form; however, the yield of this process was not 100%. We found the same pattern of ssDNA and dsDNA bands in PCR-products obtained by asymmetric PCR without any exonuclease being involved. These results will be the subject of a follow-up paper.

Fig. 2 depicts square-wave voltammograms representing the detection of the single-stranded PCR product together with different controls. The large voltammetric signal of the osmium tetroxide bipyridine-labeled PCR product (Fig. 2c) indicates not

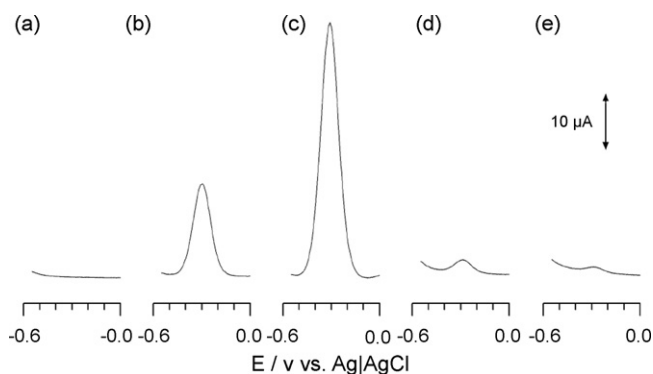


Fig. 2. Square-wave voltammograms at gold disk electrodes in Tris-buffer with immobilized probe strands, (a) *F-probe* not hybridized, (b) *F-probe* 15 min hybridized with 7.5 nmol/l $[\text{OsO}_4(\text{bipy})]$ -labeled *Fragment* at 23 °C, (c) *F-probe* 15 min hybridized with 7.5 nmol/l $[\text{OsO}_4(\text{bipy})]$ -labeled *Fragment* at 50 °C, (d) *F-probe* 15 min hybridized with 7.5 nmol/l $[\text{OsO}_4(\text{bipy})]$ -labeled *Fragment* at 23 °C, modification and hybridization without protecting strand and (e) *F-probe* 15 min hybridized with 7.5 nmol/l $[\text{OsO}_4(\text{bipy})]$ -labeled *Fragment* at 50 °C, modification and hybridization without protecting strand; frequency 200 Hz; amplitude 40 mV; stirring at 1000 rpm during hybridization.

only good hybridization efficiency with the immobilized capture probe at 50 °C, but also effective signal amplification due to multiple labeling. Each unprotected thymine base is able to react with one [OsO₄(bipy)], each delivering two electrons during the voltammetric scan. At 23 °C hybridization temperature (Fig. 2b), the signal attains less than half the value compared to 50 °C. This can probably be addressed to hindered exchange of the protective strand by the immobilized capture probe.

Fojta et al. did not include base mismatches into the protective strand, since it had been magnetically separated from the target strand after the osmium tetroxide modification [39]. However, immobilization of the target or protective strand is required instead. Future investigations will reveal how many mismatches between protective and target strand are necessary to allow easy in situ replacement of the protective strand by the immobilized probe.

The capture probe (*F-probe*) alone (Fig. 2a) did not yield any signal. Although, the unprotected and, hence, thoroughly [OsO₄(bipy)]-modified *Fragment* gave a very small signal at room temperature (Fig. 2d), this response could be significantly suppressed by applying an elevated hybridization temperature of 50 °C (Fig. 2e). Such minimal electrochemical response can probably be attributed to either unspecific adsorption of the target to the electrode surface or weak hybridization interactions between probe and target strands. In both cases, elevated temperature is expected to diminish the binding.

We further investigated the effect of hybridization temperature. Fig. 3 illustrates that elevated hybridization temperature has a positive effect up to 50 °C. At even higher temperature, the signal levels off.

In contrast, earlier investigations resulted in an optimal hybridization temperature between 30 and 40 °C [36–38]. The different behavior found in this study can probably be attributed to longer protective strands and capture probes (27 bases each, compared to 20 in the earlier studies). At temperatures higher than 70 °C, we expect disruption of the probe layer resulting at least in a loss of hybridization capability, as we observed earlier [13].

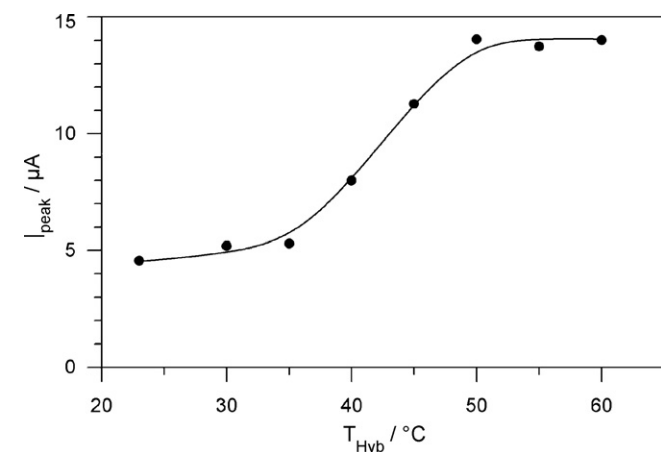


Fig. 3. Dependence of the square-wave voltammetric signals on the hybridization temperature, *F-probe*-SAM 15 min hybridized with 7.5 nmol/l target (*Fragment*). Other conditions as in Fig. 2.

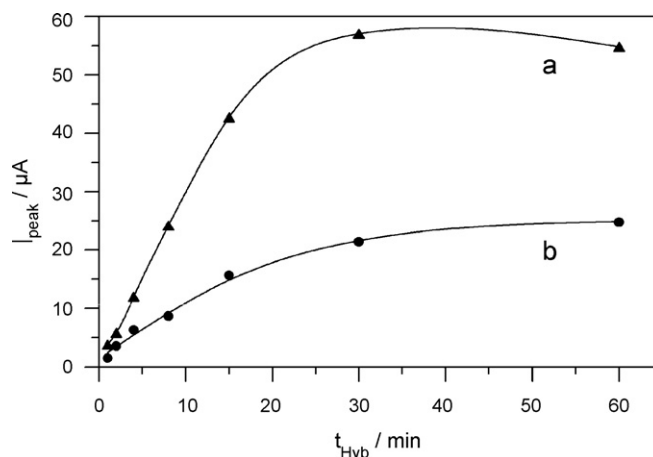


Fig. 4. Effect of the hybridization time of immobilized *F-probe* with 7.5 nmol/l target (*Fragment*) at (a) 50 and (b) 23 °C hybridization temperature. Other conditions as in Fig. 2.

Fig. 4 exhibits the effect of hybridization time upon the voltammetric signals at two different temperatures. According to Fig. 2b and c, we observed better hybridization efficiency at elevated temperature. This leads to a faster saturation of the immobilized probe layer. Even at 60 min hybridization at 23 °C, we obtained only half of the maximum signal compared to 30 min at 50 °C. Such thermal hybridization acceleration is more effective than mechanical treatment alone [38].

4. Conclusions

Single-stranded PCR products labeled with electrochemically active osmium tetroxide bipyridine can be detected voltammetrically by hybridization with probe strands immobilized at gold electrodes. Such ssDNA targets can be obtained by means of Lambda Exonuclease treatment followed by hybridization with short protective strands and covalent labeling with osmium tetroxide bipyridine. Square-wave voltammetric signals of these osmium labels are obtained only upon hybridization. An optimal 50 °C hybridization temperature has been found with a saturation of the probe layer after 30 min hybridization time at 7.5 nmol/l target concentration. The blank capture probe layer alone did not yield any signal. Unprotected strands produced almost no interference. Such double-selective switch-on electrochemical hybridization assays hold great promise for the specific detection of PCR products.

Congratulation

Gerd-Uwe Flechsig likes to congratulate Professor Joseph Wang in the occasion of his 60th birthday wishing him all the best.



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References

- [1] J. Wang, G. Rivas, J.R. Fernandes, J.L.L. Paz, M. Jiang, R. Waymire, *Anal. Chim. Acta* 375 (1998) 197.
- [2] J. Wang, A.N. Kawde, *Anal. Chim. Acta* 431 (2001) 219.
- [3] J. Wang, A.N. Kawde, A. Erdem, M. Salazar, *Analyst* 126 (2001) 2020.
- [4] J. Wang, R. Polsky, D.K. Xu, *Langmuir* 17 (2001) 5739.
- [5] J. Wang, D.K. Xu, A.N. Kawde, R. Polsky, *Anal. Chem.* 73 (2001) 5576.
- [6] T.M. Herne, M.J. Tarlov, *J. Am. Chem. Soc.* 119 (1997) 8916.
- [7] S.O. Kelley, N.M. Jackson, M.G. Hill, J.K. Barton, *Angew. Chem. Int. Ed. Eng.* 38 (1999) 941.
- [8] S.O. Kelley, E.M. Boon, J.K. Barton, N.M. Jackson, M.G. Hill, *Nucleic Acids Res.* 27 (1999) 4830.
- [9] E.M. Boon, D.M. Ceres, T.G. Drummond, M.G. Hill, J.K. Barton, *Nat. Biotechnol.* 18 (2000) 1096.
- [10] E.M. Boon, J.K. Barton, *Curr. Opin. Struct. Biol.* 12 (2002) 320.
- [11] E.L.S. Wong, F.J. Mearns, J.J. Gooding, *Sens. Actuator B: Chem.* 111 (2005) 515.
- [12] E.L.S. Wong, J.J. Gooding, *Anal. Chem.* 78 (2006) 2138.
- [13] G.-U. Flechsig, J. Peter, G. Hartwich, J. Wang, P. Gründler, *Langmuir* 21 (2005) 7848.
- [14] G.-U. Flechsig, J. Peter, K. Voss, P. Gründler, *Electrochem. Commun.* 7 (2005) 1059.
- [15] B. Meric, K. Kerman, D. Ozkan, P. Kara, S. Erensoy, U.S. Akarca, M. Mascini, M. Ozsoz, *Talanta* 56 (2002) 837.
- [16] G. Marrazza, G. Chiti, M. Mascini, M. Anichini, *Clin. Chem.* 46 (2000) 31.
- [17] M. Minunni, S. Tombelli, E. Mariotti, M. Mascini, *Fresenius J. Anal. Chem.* 369 (2001) 589.
- [18] K. Kerman, D. Ozkan, P. Kara, H. Karadeniz, Z. Ozkan, A. Erdem, F. Jelen, M. Ozsoz, *Turk. J. Chem.* 28 (2004) 523.
- [19] J. Wang, *Anal. Chim. Acta* 469 (2002) 63.
- [20] E. Palecek, F. Jelen, *Crit. Rev. Anal. Chem.* 32 (2002) 261.
- [21] J.J. Gooding, *Electroanalysis* 14 (2002) 1149.
- [22] T.G. Drummond, M.G. Hill, J.K. Barton, *Nat. Biotechnol.* 21 (2003) 1192.
- [23] P. de-los-Santos-Álvarez, M.J. Lobo-Castañón, A.J. Miranda-Ordieres, P. Tuñón-Blanco, *Anal. Bioanal. Chem.* 378 (2004) 104.
- [24] E. Palecek, M. Fojta, *Anal. Chem.* 73 (2001) 74A.
- [25] E. Palecek, *Methods Enzymol.* 212 (1992) 139.
- [26] E. Palecek, E. Lukasova, F. Jelen, M. Vojtiskova, *Bioelectrochem. Bioenerg.* 8 (1981) 497.
- [27] E. Lukasova, F. Jelen, E. Palecek, *Gen. Physiol. Biophys.* 1 (1982) 53.
- [28] E. Palecek, M.A. Hung, *Anal. Biochem.* 132 (1983) 236.
- [29] E. Lukasova, M. Vojtiskova, F. Jelen, T. Sticzay, E. Palecek, *Gen. Physiol. Biophys.* 3 (1984) 175.
- [30] E. Palecek, M. Fojta, F. Jelen, *Bioelectrochemistry* 56 (2002) 85.
- [31] M. Fojta, L. Havran, S. Billova, P. Kostecka, M. Masarik, R. Kizek, *Electroanalysis* 15 (2003) 431.
- [32] M. Fojta, L. Havran, M. Vojtiskova, E. Palecek, *J. Am. Chem. Soc.* 126 (2004) 6532.
- [33] P. Kostecka, L. Havran, H. Pivonkova, M. Fojta, *Bioelectrochemistry* 63 (2004) 245.
- [34] B. Yosypchuk, M. Fojta, L. Havran, M. Heyrovsky, E. Palecek, *Electroanalysis* 18 (2006) 186.
- [35] G. Wang, L. Wu, X. Wang, J. Cheng, W. Yang, *Int. Patent Application WO 02/16647 A1* (2000).
- [36] G.-U. Flechsig, T. Reske, *Patent DE 10 2005 039 726 B3* (2005).
- [37] G.-U. Flechsig, T. Reske, *Anal. Chem.* 79 (2007) 2125.
- [38] J. Peter, T. Reske, G.-U. Flechsig, *Electroanalysis* 19 (2007) 1356.
- [39] M. Fojta, P. Kostecka, M. Trefulka, L. Havran, E. Palecek, *Anal. Chem.* 79 (2007) 1022.

Review

Carbon nanotubes for electrochemical biosensing

Gustavo A. Rivas^{a,*}, María D. Rubianes^a, Marcela C. Rodríguez^a, Nancy F. Ferreyra^a,
Guillermina L. Luque^a, María L. Pedano^a, Silvia A. Miscoria^a, Concepción Parrado^b

^a INFIQC, Departamento de Físico Química, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria,
5000 Córdoba, Argentina

^b Departamento de Química Analítica, Facultad de Química, Universidad Complutense de Madrid, Madrid, Spain

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Abstract

The aim of this review is to summarize the most relevant contributions in the development of electrochemical (bio)sensors based on carbon nanotubes in the last years.

Since the first application of carbon nanotubes in the preparation of an electrochemical sensor, an increasing number of publications involving carbon nanotubes-based sensors have been reported, demonstrating that the particular structure of carbon nanotubes and their unique properties make them a very attractive material for the design of electrochemical biosensors.

The advantages of carbon nanotubes to promote different electron transfer reactions, in special those related to biomolecules; the different strategies for constructing carbon nanotubes-based electrochemical sensors, their analytical performance and future prospects are discussed in this article.

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* Corresponding author. Tel.: +54 351 4334169/80; fax: +54 351 4334188.

E-mail address: grivas@mail.fcq.unc.edu.ar (G.A. Rivas).

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The aim of this review is to summarize the most relevant contributions in the development of electrochemical (bio)sensors based on carbon nanotubes in the last 5 years. The work is mainly focused on amperometric and voltammetric sensors, although some examples dealing with chronopotentiometric transduction are also included.

1. Carbon nanotubes: general considerations

Nanomaterials have received great attention in recent years in different fields due to their enormous potential. Among them, carbon nanotubes (CNT), discovered in 1991 by Iijima [1], represent an important group of nanoscale materials. They possess one of the simplest chemical composition and atomic bonding configuration, and show the most extreme diversity and richness among nanomaterials referred to structure and associated properties [2]. Since their discovering, CNT have been the goal of numerous investigations due to their unique structural, electronic and mechanical properties that make them a very attractive material for a wide range of applications [1,3–7].

Carbon nanotubes are built from sp^2 carbon units and present a seamless structure with hexagonal honeycomb lattices, being several nanometers in diameter and many microns in length [3,6]. CNTs are closed structures that present two well defined regions with clearly different properties, the tube and the cap, which is half-fullerene-like molecule with topological defects that in this case are mainly pentagons (Fig. 1) [6,8,9].

Basically, there are two groups of carbon nanotubes, multi-wall (MWCNTs) and single-wall (SWCNTs) carbon nanotubes [3,6,10,11]. MWCNTs, can be visualized as concentric and closed graphite tubules with multiple layers of graphite sheets that define a hole typically from 2 to 25 nm separated by a distance of approximately 0.34 nm [2–4]. SWCNTs consist of a single graphite sheet rolled seamlessly defining a cylinder of 1–2 nm diameter. There are three basic methods for synthesis of SWCNTs and MWCNTs: electrical arch discharge, laser ablation (laser vaporization) and chemical vapor deposition (CVD) (or catalytic decomposition of hydrocarbons) [1,7,9,10,12,13].

2. Carbon nanotubes as electrode material

CNTs possess interesting electrochemical properties. Several works have established that the electroactivity of CNTs is due to the presence of reactive groups on the surface [10,14,15].

Ab-initio calculations demonstrated that the improvement in the electron transfer is due to the curvature of the tubes that originate changes in the energy bands close to the Fermi level [15]. The presence of pentagonal defects produce regions with charge density higher than those observed in the region of hexagonal graphite, either in planar or in tubular structures demonstrating the connection between topological defects and CNTs electroactivity [15]. In this sense, Compton's group reported a very interesting work dealing with the investigation about the reason why CNTs present the enhanced electrocatalytic activity and proposed that this activity is due to the presence of edge-plane like sites located at the end and in the “defects” areas of the tubes [16]. An interesting electrocatalytic activity is obtained in the presence of CNT, associated with the CNT dimensions, electronic structure and topological defects present on the tube surface [7,11,14–16]. In general, lower overvoltages and higher peak currents are observed in the voltammetric response of several molecules at electrodes modified with CNTs. Due to these unique properties, CNTs have received enormous attention for

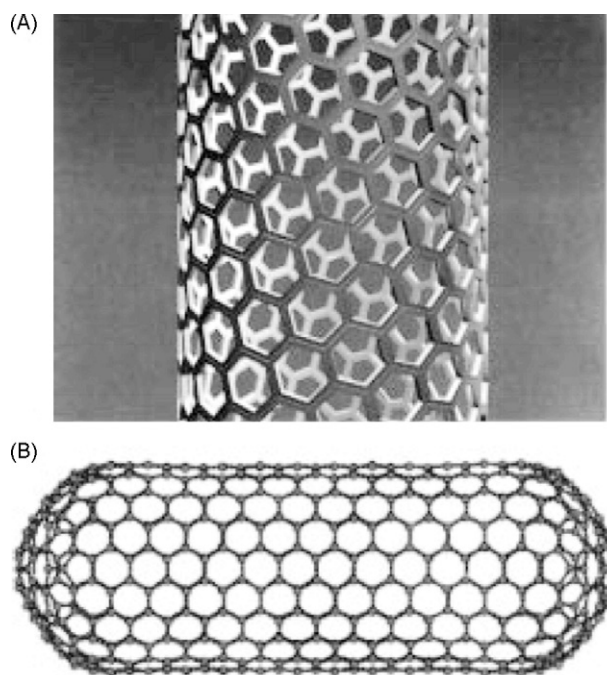


Fig. 1. (A) Scheme of the basic unit of a carbon nanotube. (B) Scheme of a carbon nanotube showing the two regions, the cap and the tube. Adapted from reference [6].

the preparation of electrochemical sensors, as it was largely reviewed [17–24].

In general, some pretreatment of the CNTs is necessary to eliminate metallic impurities, and/or to improve the electron transfer properties and/or to allow further functionalization. The protocols are based on the oxidation of CNTs under different conditions. In all cases the ends and side-walls become rich in oxygenated functions, mainly carboxylic groups. Depending on how drastic is the treatment, it is possible not only to increase the density of oxygenated functions but also to break the tubes or even to shorten them. Palleschi and co-workers [25] proposed the oxidation at 400 °C under an air flow. Sotiropoulou and Chaniotakis [26] proposed the activation by air oxidation at 600 °C under air flow.

Activation by treatment in acidic solutions have been widely used. Solutions of sulfuric [27]; nitric [28–32]; and hydrochloric [25,33] acids, either concentrated or diluted, alone or mixed [26,32] have been used at room temperature or under refluxing, with or without sonication for different times. Pumera [34] reported an interesting work where demonstrates that even after prolonged washing of CNTs in nitric acid at elevated temperatures, some residual metal catalyst nanoparticles remain intercalated within the nanotube channel or in the bamboo segment or as graphene sheath protected nanoparticles.

Electrochemical treatments have been also employed, either potentiostatic or potentiodynamic, depending on the system under investigation. In some cases, the pretreatments were based on a combination of different chemical and electrochemical protocols [35,36].

2.1. Strategies for the preparation of carbon nanotubes-based electrodes

One of the problems for the preparation of sensors based on the use of carbon nanotubes is their insolubility in usual solvents. Therefore, several strategies have been proposed for the immobilization of CNT on electrochemical transducers, like dispersion in different solvents or polyelectrolytes or incorporation in composite matrices using distinct binders.

2.1.1. Dispersion of carbon nanotubes in solvents and polyelectrolytes

The preparation of an electrochemical sensor modified with a CNT-dispersion basically consists of casting the electrode, usually glassy carbon or gold, with a drop of the given dispersion, followed by a drying step under different conditions.

One alternative to obtain an electrochemical sensor based on CNT is by casting a polished glassy carbon electrode (GCE) with a dispersion of MWCNTs in concentrated sulfuric acid (1 mg/mL) followed by drying at 200 °C for 3 h and careful rinsing [27]. Wang et al. [37] proposed the preparation of MWCNT–GCE by casting a polished GCE with a concentrated nitric acid solution containing 2 mg/mL MWCNTs followed by a drying step at room temperature for 30 min.

Li and co-workers [38,39] described the preparation of CNTs-modified GCE by casting the GCE with a suspension of SWCNTs in dimethylformamide (DMF). The drying step was

performed under an IR heat lamp. A similar protocol was used by Pang and co-workers [40], although the dispersion was 2 mg/mL in CNT.

The ability of the perfluorosulfonated polymer Nafion to disperse SWCNTs and MWCNTs was proposed by Wang et al. [41]. A homogeneous, well-distributed dispersion of Nafion/CNTs was obtained with 0.5% (v/v) (in 0.05 M phosphate buffer pH 7.4) and 5% (v/v) polymer solution for SWCNTs and MWCNTs, respectively. Mao and co-workers [29] reported the use of a CNT-modified GCE prepared by casting the GCE with a CNTs dispersion prepared in 0.5% methanolic Nafion solution and followed by the evaporation of the solvent. Rivas et al. [42] proposed the use of a MWCNT–Nafion dispersion dropped onto a glassy carbon electrode as a platform for further self-assembling of polyelectrolytes.

The biopolymer chitosan (CHIT) was also used for solubilizing MWCNT [43,44]. In some cases after dropping the dispersion on the electrode surface, an additional pretreatment was also performed, either in NaOH or in glutaraldehyde in order to stabilize the resulting layer.

Rubianes and Rivas proposed the highly efficient dispersion of MWCNT by the polycation polyethylenimine (PEI) as well as the excellent performance of GCE modified with this dispersion, not only in batch [45] but also as detector in flow injection analysis and capillary electrophoresis [46]. Rivas' group also proposed the use of polylysine as efficient dispersing agent of MWCNT due to the large amount of amine residues that facilitate the interaction with the MWCNT [47].

Tkac and Ruzgas [48] made an interesting comparison about the influence of the dispersing agent (DMF, cyclohexanone, chitosan and Nafion) and dispersing conditions on the response of the resulting electrode. They found that charged polymers are more efficient than organic solvents to disperse CNTs, being chitosan the one that offers the best response once cast on the electrode surface (using hydrogen peroxide as marker).

The successful use of surfactants for dispersing CNT has been also reported. Hu and co-workers [49] proposed an amperometric sensor by casting a GCE with a dispersion of MWCNTs in water in the presence of dihexadecylhydrogenphosphate (DHP). Zhang and Gao [50] reported the use of sodium dodecyl sulfate as a dispersing agent for oxidized MWCNT.

Another avenue for immobilizing CNTs was the dispersion of CNTs in β -cyclodextrin (CD) (2% aqueous solution, CNT 0.1 mg/mL), as it was proposed by Wang Z. et al. [51].

Compton and co-workers [52] proposed the immobilization of CNT on basal plane pyrolytic graphite electrodes (BPPG) by dispersing the powder in acetonitrile, casting the electrode and eliminating the solvent by evaporation. Another methodology consisted of the preparation of MWCNTs-modified electrode by casting a gold surface with a suspension of CNTs prepared by dispersing the oxidized MWCNTs in double distilled water (0.5 mg/mL) [30] and drying under vacuum at about 50 °C.

2.1.2. Incorporation of carbon nanotubes within composite matrices using different binders

As it is widely known, composite materials present a large number of advantages over other materials. For that reason,

composites based on the use of CNT have received enormous attention.

Teflon was successfully used as binder for dispersing MWCNT and preparing composite CNT-based electrodes [53,54]. Hill and co-workers [55] showed the feasibility to use CNT as electrode material, by packing oxidized CNTs into a glass capillary in bromoform, nujol, deionized water or mineral oil. Britto et al. [56] proposed the construction of electrodes by dispersing CNTs in bromoform. Rivas and Rubianes [57,58] reported for the first time a composite material prepared in an easy, fast and very efficient way by dispersing MWCNTs within mineral oil (60.0/40.0%, w/w CNT/oil). The resulting carbon nanotube paste electrode (CNTPE) retains the properties of the classical graphite carbon paste electrode (CPE) and those of the composite nature. CNTPE prepared with short (1–5 μm length) and long MWCNTs (5–20 μm length) of 20–50 nm diameter demonstrated to be highly useful as detectors in flow systems [59]. Chicharro et al. [60] also used electrochemically activated CNTPE for the highly sensitive detection of amitrole. Palleschi and co-workers [25] reported a composite electrode obtained by mixing SWCNTs and mineral oil, 60/40% (w/w) (CNT/oil). The use of a CNTPE prepared by mixing MWCNTs of 5–20 nm length with oil in a ratio 3:2 (CNT/oil) was also proposed [61]. Magno and co-workers [62] described an amperometric biosensor prepared by mixing MWCNTs with mineral oil and coating by a polymer obtained by electropolymerization of 3,4-dihydroxybenzaldehyde. Wang et al. [63] reported the determination of carbohydrates by capillary electrophoresis using a CNTPE containing Cu as detector prepared by hand mixing mineral oil, MWCNT and copper powder in a weight ratio 1:1:2 (carbon/oil/Cu). Rivas and co-workers [64,65] proposed the use of CNTPE modified with Cu microparticles for the highly sensitive detection of amino acids, albumin and glucose. Fabregas and co-workers [66] reported the analytical applications of screen printed modified with MWCNT/polysulfone.

Another interesting strategy proposed by Wang and Musameh [67] was the preparation of a composite material based on the dispersion of CNTs in ink, in a way similar to that for preparing the classical graphite screen printed electrodes (SPE). The electrodes were fabricated following two strategies, using the same ink as the one for preparing the SPE, and mixing 60 mg CNTs with 500 μL of isophorone solution containing different compounds until homogeneous aspect. The ink was then printed on alumina ceramic plates and the resulting electrodes were cured for 1 h at 150 $^{\circ}\text{C}$ and then allowed to cool down at room temperature. SEM pictures showed a microporous structure of flake-shaped particles non-uniformly distributed.

2.1.3. Other methodologies

Compton and co-workers [28] proposed the immobilization of MWCNTs on BPPG by abrasively attaching them on the electrode surface. Luo and co-workers [33] reported the intercalation of CNTs in a pyrolytic graphite electrode previously polished with emery paper and alumina slurries and sonicated with water.

One interesting work was presented by Liu and co-workers [68] who proposed the assembling of oxidized

SWCNTs on the top of a gold surface modified with monolayers of 11-amino-*N*-undecylmercaptan through the carboxylic residues using dicyclohexylcarbodiimide as condensing agent.

Another interesting avenue was the use of MWCNTs arrays grown by CVD using Fe as catalyst and C_2H_4 followed by the use of a film of spin-on glass (SOG) to fill the gaps between the individual CNTs to provide the structural support to the CNTs at the same time that serve as a dielectric material insulating the individual CNTs [69].

The self-assembling layer-by-layer (L-b-L) of polyelectrolytes on previously functionalized CNTs was also proposed [70]. In one case, the negative charges were originated onto the surface of carbon nanotubes by adsorbing a pyrene derivative, which serves as platform for further assembling of poly(diallyldimethylammonium) (PDDA) and polystyrene sulfonate (PSS).

Guo and co-workers [71] reported the non-covalent modification of MWCNTs by using an innovative scheme. The carboxylic groups of oxidized SWCNTs were first converted to acylchloride in SOCl_2 and then reacted with didecylamine. After extracting the nanotubes, removing the solvent and dispersing them in toluene, they were allowed to react with the interlinker 17-(1-pyrenyl)-13-oxo-heptadecanethiol (PHT). The PHT binds on the surface of SWCNT mainly through π – π interactions between the pyrenyl units of PHT and the side-wall of modified SWCNT. Since the PHT presents a thiolated residue, the gold nanoparticles can be bound to the architecture and MWCNT densely coated with gold nanoparticles can be obtained, converting this multistructure in a good platform for further biosensors designs.

3. Use of electrodes based on carbon nanotubes for sensing different analytes

3.1. Catecholamines and related compounds

Britto et al. [56] reported a significant improvement in the electrochemical behavior of dopamine using a composite prepared by dispersion of CNTs in bromoform, with peak potential separation of 30 mV. Rubianes and Rivas [57] demonstrated the advantages of a composite material prepared by mixing MWCNTs and mineral oil (CNTPE) on the electrochemical behavior of different biomolecules. The voltammetric signals for dopamine, ascorbic acid, dopac and uric acid largely improved at the composite containing CNTs. The peak potential separation for dopamine and dopac decreased 133 and 313 mV at CNTPE compared to CPE, while the overvoltages for the oxidation of ascorbic acid and uric acid decreased 230 and 160 mV, respectively. CNTPE demonstrated to be highly efficient as detector for flow injection and capillary electrophoresis determinations of dopamine and related compounds [59]. Rivas' group [72] proposed the use of a GCE modified with a dispersion of CNT in PEI for the selective determination of dopamine, even in large excess of ascorbic acid and serotonin through the facilitated electron transfer in the presence of CNT that allows an effective resolution of both processes. A significant improvement in the redox

behavior of dopamine, 5-hydroxy-tryptamine and other compounds at CNTPE was reported by Palleschi and co-workers [25].

Chung and co-workers [73] reported a needle-type biosensor to determine dopamine and glutamate obtained by attachment of MWCNT to the end of an etched tungsten tip. Luo and co-workers [33] described the simultaneous differential pulse voltammetry detection of dopamine, ascorbic acid and serotonin using an electrode based on the incorporation of CNTs, with successful application for the determination of dopamine and serotonin in rabbit's brain.

The study of the groups involved in the redox behavior of CNTs as well as the electrochemical behavior of dopamine and related compounds at the electrodes containing CNT was also reported [74]. XPS and IR analysis showed the participation of the carboxylic groups in the redox behavior of the SWCNT–GCE, which were reduced to CH_2OH via four electrons. An important shifting of the dopamine oxidation peak potential was observed at SWCNT-film modified GCE. A linear relationship between current and dopamine concentration was obtained from 1.0×10^{-6} to 2.0×10^{-4} M.

Li and co-workers [38] reported the voltammetric detection of dopac at SWCNTs-modified GCE in the presence of 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 5-hydroxy-tryptamine as well as the electrocatalytic oxidation of norepinephrine with detection limit of 6.0×10^{-6} M [75]. Pang and co-workers [40] showed a linear range between 5.0×10^{-7} and 2.0×10^{-5} M L-dopa and a detection limit of 3.0×10^{-7} M. The electrocatalytic activity of the SWCNTs-modified-GCE was also demonstrated with dopamine, epinephrine and ascorbic acid.

An enhancement in the response of dopamine and an effective discrimination against uric acid and ascorbic acid was obtained with glassy carbon modified with a dispersion of SWCNT non-covalently functionalized with Congo red [76]. Yang and co-workers [77] proposed the immobilization of MWCNT by L-b-L self-assembling of polyelectrolytes on a screen printed electrode for the detection of ascorbic acid at potentials 350 mV less positive than at the bare electrode.

Compton and co-workers [28] proposed the detection of epinephrine using MWCNTs abrasively attached to the basal plane pyrolytic graphite (BPPG). The electrode demonstrated a good performance, with a decrease of 300 mV in the oxidation overvoltage for epinephrine and a significant increase in the associated peak current that makes possible to detect $0.02 \mu\text{M}$. Wang et al. [78] proposed an electrochemical transducer for the detection of dopamine based on the immobilization of Nafion/SWCNT on a poly(3-methylthiophene)-modified glassy carbon electrode, with a detection limit of 5.0 nM .

3.2. Homocysteine and related compounds

Wang and co-workers [61] proposed the use of CNTPE for the detection of homocysteine with detection limits at micromolar levels. A CNT/Nafion modified-GCE was used for the amperometric determination of homocystein with a detection limit of 6.0×10^{-2} M [29].

3.3. Carbohydrates

MWCNT composite electrodes containing Cu were proposed as detectors for capillary electrophoresis determination of carbohydrates using NaOH solution pH 12.5 [63]. Xia and co-workers [79] showed an amperometric biosensor for the nonenzymatic detection of glucose in alkaline solution based on the use of a GCE modified with Pt nanoparticles supported on CNTs and Nafion. A highly sensitive and selective nonenzymatic glucose sensor was also obtained by deposition of copper onto CNT-modified GCE and further determination in alkaline solutions [80].

3.4. Nitroaromatic compounds

Nanocomposites of SWCNT and MWCNT and metal nanoparticles (Pt, Cu, Au) solubilized in Nafion were used to modify GCE to allow the sensitive and selective quantification of nitroaromatics [81]. The best electrode was obtained from the association of Cu nanoparticles and SWCNT “solubilized” in Nafion.

3.5. NADH

Wang and co-workers [27] reported a decrease of 490 mV (compared to GCE) in the oxidation overvoltage for NADH at GCE coated with MWCNTs allowing the fast and stable detection of NADH at low potentials. Wang et al. [67] also showed the advantages of CNTs–SPE for the oxidation of NADH. The accelerated electrooxidation of NADH at CNT/Teflon biocomposite was also described [53].

Rubianes and Rivas [82] reported that the oxidation of NADH at CNTPE starts at -0.100 V, that is, 0.300 V less positive than at CPE, due to the catalytic effect of carbon nanotubes (Fig. 2A). As it is shown in Fig. 2B, CNTPE showed an effective short-term stability since even after 15 min at 0.400 V, the oxidation signal of 1.0×10^{-5} M NADH decreased less than 20% (similar experiments at CPE showed a decrease in the signal of around 80%). The dispersion of CNTs with chitosan covalently attached to the redox mediator Azure dye [83] and the assembling of polyaniline onto commercially available poly(aminobenzenesulfonic acid)-modified SWCNT allowed important decreases in the overvoltage for the oxidation of NADH [84]. Dong and co-workers [85] reported a significant improvement in the NADH oxidation by using a supramolecular multistructure obtained by deposition of PDDA, PSS and the MWCNT/PDDA dispersion onto negatively charged ITO surfaces. The authors reported a decrease in the overpotential for NADH oxidation with the number of bilayers and a detection limit of $6 \mu\text{M}$ at 0.4 V.

3.6. Amino acids and proteins

Wang and co-workers [86] proposed the amperometric detection of non-electroactive amino acids at CNTs–GCE and at Ni–CNTs/Nafion–GCE in a NaOH solution at 0.55 V. The authors performed an interesting comparison between the response of electrodes using SWCNT and MWCNT synthesized

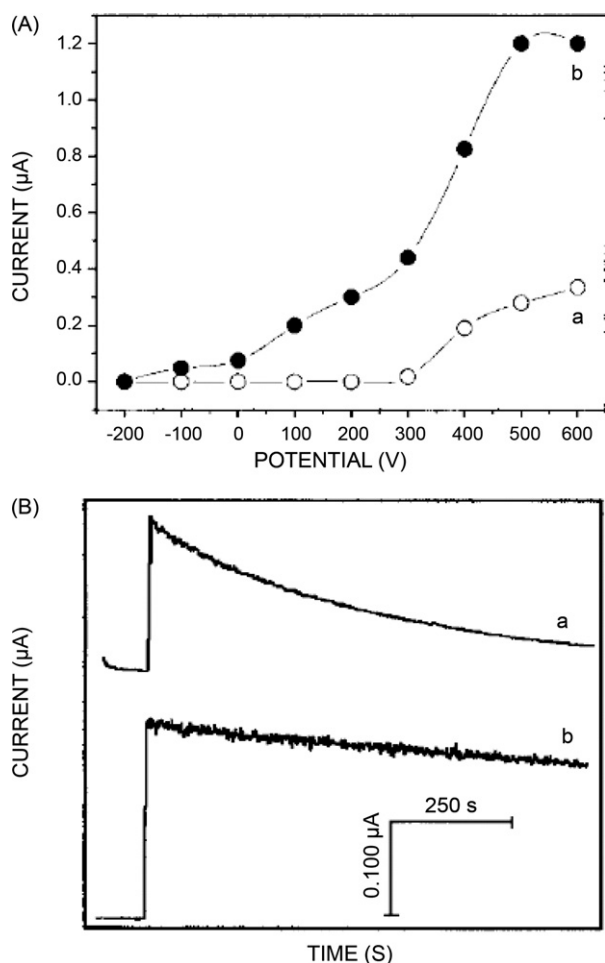


Fig. 2. (A) Hydrodynamic voltammograms for 1.0×10^{-2} M ethanol at CPE-NAD⁺-ADH (a) and at CNTPE-NAD⁺-ADH (b); (B) amperometric recordings at CPE (a) and CNTPE (b) after one addition of 1.0×10^{-5} M NADH. Working potential: 0.400 V. Adapted from reference [82] Rubianes and Rivas, *Enzymatic Biosensors Based on Carbon Nanotubes Paste Electrodes*, *Electroanalysis* 17 (2005) pp. 73–78. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

by ARC or CVD, demonstrating that electrodes prepared by CVD are the most active. In the case of Ni-CNTs-GCE, an additional layer of nickel hydroxide electrogenerated improved even more the amperometric response of different amino acids by complex formation, with detection limits in the order of 10^{-5} M.

The incorporation of copper into the CNTPE is another interesting strategy for the design of amino acids and albumin sensors based on the facilitated copper oxidation due to the complex formation with the amino acid [64]. The amperometric response at 0.000 V at CNTPE-Cu (6.0%, w/w) for successive additions of 5.0×10^{-4} M L-histidine showed a sensitive and fast response, as a consequence of the complex formation between Cu(II) produced at the applied potential and L-histidine. Similar results were obtained with other amino acids. In this way, the incorporation of copper within CNTPE allowed the detection of amino acids, electroactive or not, at very low potentials and at physiological pHs. Based on these properties, the electrode was also used for the square wave voltammetric detection of albumin [64]. When the metal is not present, only the direct oxidation of tyro-

sine and tryptophan residues at around 0.7 V can be observed. When CNTPE is modified with copper, besides the direct amino acids oxidation, there was a huge peak at -0.100 V due to the facilitated oxidation of copper in the presence of the amino acid residues. The methodology was validated using the classical spectrophotometric determination, with excellent agreement.

An obvious enhancement in the electrooxidation signals of L-tyrosine, L-cysteine and L-tryptophan was obtained at SWCNT-arrayed electrodes on Pt surfaces obtained by direct growth of SWCNT by CVD on Pt electrodes [87].

Wang and Musameh [88] showed that the modification of GCE with MWCNT/DMF, allows the accelerated electron transfer of insulin and the consequent highly sensitive and stable determination at physiological pHs. Wang's group also demonstrated [89] that GCE modified with ruthenium oxide (by electrodeposition from RuCl₃/HClO₄ solution) and CNT (by dropping a dispersion of MWCNT/DMF) makes possible an important improvement in the insulin quantification. The authors attributed this enhancement to the synergistic effect of CNT and ruthenium oxide layers.

3.7. Uric acid

A selective response for uric acid, even in the presence of ascorbic acid, was obtained at β -cyclodextrin-CNT-modified-GCE [51]. Rivas and co-workers proposed the highly sensitive and selective uric acid quantification at GCE modified with MWCNT/polylysine in the presence of large excess of ascorbic acid following two strategies [90]. One of them was based on the direct determination of uric acid in the presence of large excess of ascorbic acid. The other one relied on the selective preconcentration of uric acid at GCE/MWCNT/polylysine followed by the sensitive detection after medium exchange due to the almost negligible adsorption of ascorbic acid.

3.8. Estrogens

Yáñez-Sedeño and co-workers [91] proposed an enhancement in the LC-EC detection of phenolic estrogens at GCE modified with MWCNT. Another avenue was the use of the surfactant cetyltrimethylammonium bromide to improve the response of estradiol at a GCE modified with MWCNT/Congo red dispersion due to the hydrophobic interaction with MWCNT that allows obtaining not only higher sensitivity but also antifouling properties in the resulting electrode [92].

4. Use of carbon nanotubes for the development of electrochemical biosensors

A biosensor is a device constituted by a biorecognition layer encharged of the biomolecular recognition of the analyte and a transducer that is responsible for the conversion of the biorecognition event into a useful electrical signal [93]. Enzymes, antigens, antibodies, nucleic acids, receptors and tissues have been used as biorecognition elements. According to this element, it is possible to divide biosensors in enzymatic (involving a biocatalytic event) and affinity (involving an affin-

ity event) biosensors [94]. The nature of the electrode is very important for the electrochemical transduction process. In this sense, CNTs represent an important alternative for the transduction event due to their excellent electronic properties. In fact, recent years have witnessed the development of highly sensitive and selective electrochemical biosensors based on the use of carbon nanotubes.

4.1. Carbon nanotubes-based-electrochemical enzymatic biosensors

4.1.1. General aspects

Since the first enzymatic electrode proposed by Clark and Lyons more than 40 years ago, electrochemical biosensors based on the use of enzymes have received considerable attention due to the advantages of the association of the biocatalytic activity of enzymes with the high sensitivity and versatility of the electrochemical transduction. The enzyme immobilization step is critical, since the biocatalyst has to remain active to perform an efficient biorecognition of the substrate. The other aspect to consider is that the transducer where the enzyme is immobilized has to allow a fast charge transfer to ensure a rapid and sensitive response. Several strategies for immobilizing proteins on CNTs modified electrodes have been proposed, the ones involving non-covalent functionalization of the sidewalls of SWCNTs being the best to preserve the sp^2 CNT structure and their electronic characteristics.

Sun et al. [95] proposed a very interesting discussion about the adsorption of proteins on CNTs using ferritin as a model. Davis and co-workers [96] reported an important work about the

immobilization of ferritin, cytochrome *c* and glucose oxidase (GOx) on oxidized, purified and vacuum-annealed SWCNTs in aqueous solution. Dai and co-workers [97] presented a very attractive work about non-covalent functionalization of CNTs sidewalls for the efficient immobilization of ferritin, streptavidin and biotinyl-3,6-dioxaoctanediamine. The first step was the non-covalent functionalization of SWCNTs by irreversible adsorption of a bifunctional molecule, 1-pyrenebutanoic acid, succinimidyl ester onto the hydrophobic surfaces of SWCNTs dispersed in DMF or methanol. This molecule interacts with the basal plane of graphite via π -stacking through the aromatic rings with the sidewalls of SWCNTs. The succinimidyl residues are highly reactive to nucleophilic substitution by primary and secondary amines of proteins or other molecules making possible, in this way, the immobilization of proteins.

Gooding et al. [98] presented a strategy for studying the electron transfer properties of redox enzymes like microperoxidase 11 attached to the end of aligned SWCNTs. A cysteamine-derivatized gold electrode was immersed in a dispersion of shortened SWCNTs in DMF containing dicyclohexylcarbodiimide to convert the carboxyl group located at the end of shortened CNTs into active carbodiimide esters. The SWCNTs were aligned normal to the electrode surface. Finally, microperoxidase was attached to the free ends of the tubes by incubation in a microperoxidase solution at 4 °C overnight. Fig. 3 shows a scheme of this procedure.

SWCNT normal aligned can act as molecular wires allowing the electrical communication between the electrode and redox proteins covalently attached to the ends of SWCNTs.

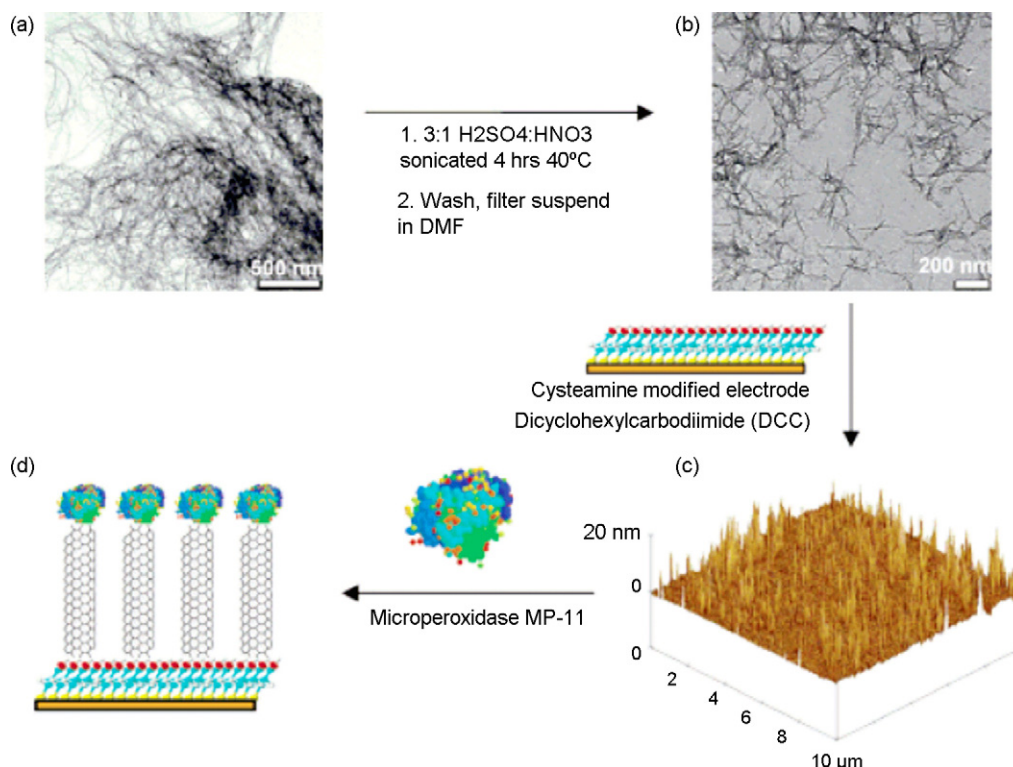


Fig. 3. Scheme of the different steps involved in the fabrication of aligned shortened SWCNT arrays for direct electron transfer of microperoxidase MP-11. Adapted from reference [98].

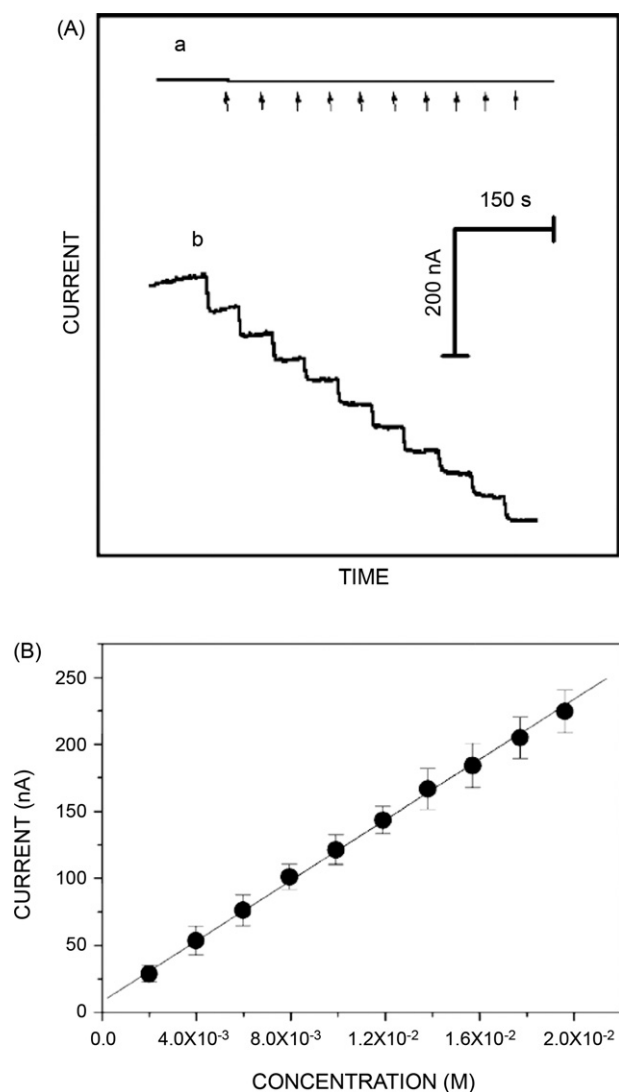


Fig. 4. (A) Amperometric recordings obtained at CPE-GOx (a) and at CNTPE-GOx (b) for successive additions of 5 mM glucose. (B) Calibration plot obtained from amperometric recording for successive additions of 2 mM glucose. Working potential: -0.100 V. Adapted from reference [57].

4.1.2. Different strategies for the development of carbon nanotubes-based-electrochemical enzymatic biosensors

4.1.2.1. Glucose biosensors. Glucose biosensors have received enormous attention, mainly due to the requirement of new developments for the diagnostic and control of diabetes. Different strategies for the preparation of glucose biosensor involving CNTs have been proposed, and the most representative are included below.

Rubianes and Rivas [57] showed the advantages of using CNTPE modified with GOx for developing highly sensitive glucose biosensors without redox mediators, metals or anti-interferents layers. Fig. 4A shows the amperometric recordings obtained at -0.100 V at CPE-GOx (a) and CNTPE-GOx (b) for successive additions of 2.0 mM glucose, and Fig. 4B shows the corresponding calibration plot. Almost no response is observed at the graphite composite electrode. On the contrary, a fast and sensitive response is observed at CNTPE-GOx due to the

catalytic activity of CNTs towards hydrogen peroxide. The sensitivity obtained with CNTPE-GOx was 43 times higher than the one obtained with the CPE-GOx, with a linear range from 2.0 to 25.0 mM glucose, and a negligible interference even for large excess of ascorbic acid, uric acid and acetaminophen. In order to obtain a rigid and renewable biosensor, Alegret and co-workers [99] proposed the immobilization of GOx on a CNT epoxy-composite matrix prepared by dispersion of MWCNT inside of the epoxy resin.

The excellent thermal stability and the feasibility to monitor glucose under severe oxygen deficiency was proposed for a biosensor obtained by dispersing GOx directly within CNTs and graphite, packing the resulting biocomposite into a 21-gauge needle, and coating with 1% Nafion solution [100]. The use of a soluble CNT/poly(vinyl alcohol) nanocomposite for developing a glucose biosensor using the thick film technique was also described [101]. MWCNT composite was also prepared using Pt-NP doped sol/gel solution as a binder [102] based on the stabilization of Pt-NP by the amine groups of sol/gel. The incorporation of Pt allowed the enhancement of the sensitivity almost 4 times.

A fast, sensitive and selective response to glucose was obtained by using an electrode prepared by the incorporation of Pt-NP in SWCNTs-Nafion film on glassy carbon and carbon fiber electrodes followed by the immobilization of GOx and cross-linking with glutaraldehyde [103].

Salimi et al. [104] showed a glucose biosensor obtained by abrasive immobilization of CNTs at a BPPG according to the methodology previously described [28] followed by immobilization of a sol-gel composite solution containing GOx. Chem and co-workers [105] presented an amperometric biosensor based on the adsorption of GOx on Pt-NP-CNT/graphite and coating with a final layer of Nafion. The practical application for glucose determination in blood samples demonstrated good correlation with clinical values.

The preparation of a glucose biosensor by co-deposition of Pd and GOx onto a CNT/Nafion/electrode allowed a very sensitive glucose biosensor through the efficient oxidation and reduction of the enzymatically generated hydrogen peroxide [106]. GOx was also immobilized on Pt modified with chemically oxidized SWCNTs through covalent attachment using EDC [107]. The use of MWCNT/Nafion/GOx nanobiocomposite-modified GCE gives place to a fast and sensitive glucose biosensing [108].

Another avenue was the immobilization of GOx at CNT/Nafion-GCE by dipping the electrode in a solution containing GOx and glutaraldehyde, followed by successive immersions in a Nafion solution [41]. The important electrocatalytic activity of CNTs towards the reduction of hydrogen peroxide allowed the very sensitive and selective glucose quantification at -0.050 V. An amperometric glucose biosensor based on the use of a MWCNT-electrode modified with electrogenerated poly(neutral red) and the immobilization of GOx in the presence of glutaric dialdehyde was also described [109]. Schmidtke and co-workers [110] proposed a glucose biosensor by cross-linking of GOx with SWCNT and poly[(vinylpyridine)Os(bipyridyl)₂Cl^{2+/3+}] polymer film following two alternatives: depositing first the SWCNT on the top of GCE and then the hydrogel containing

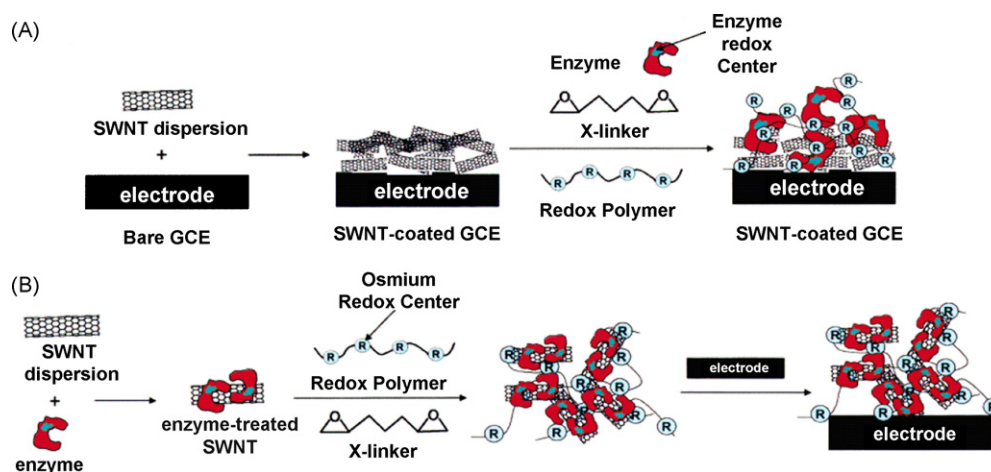


Fig. 5. Schematic representation of a biosensor following two protocols: (A) the film of SWCNT was first cast on the bare glassy carbon electrode and after drying, the redox hydrogel was cast on the top of the SWCNT-coated electrode; (B) SWCNTs were first incubated with the enzyme solution before incorporating into the redox hydrogel and then cast on a top of a bare glassy carbon electrode. Adapted from reference [110].

the redox polymer and the enzyme; or incorporating the redox polymer to a composite of GOx and SWCNTs and then modify the GCE (Fig. 5). Under these conditions the glucose oxidation signal largely increased due to a better electrical connection. A selective glucose biosensing was also obtained by covalent immobilization of GOx on CNT nanoelectrodes [111,112].

Willner's group proposed an attractive approach for the construction of a glucose biosensor based on the use of CNTs [113]. One end of oxidized SWCNTs was covalently attached to a thiolated gold surface through the generated carboxylic functions, while the other end of the SWCNT was covalently attached to FAD. The glucose biosensor was obtained in the presence of the apo-GOx, through the reconstitution of the enzyme on the edges of SWCNTs.

Following a similar strategy, Gooding and co-workers [114] showed an interesting comparison between the rate constants for GOx charge transfer when GOx is covalently attached at the end of Au/cysteamine/SWCNT and when FAD is incorporated at the end of the Au/cysteamine/SWCNT followed by further *in situ* enzyme reconstitution in the presence of the apo-GOx (Fig. 6). A rate constant 30-fold higher was reported when the apoenzyme is refolded around the surface FAD.

Chen and co-workers [115] proposed the self-assembling of MWCNT and GOx on the surface of a graphite disk, the maximum response being obtained with six MWCNT/GOx bilayers. The L-b-L technique was also used for the development of a reproducible, stable and selective amperometric glucose biosensor by the self-assembling of GOx on a GCE modified with negatively charged CNT and PDDA [116]. The use of gold modified with 3-mercaptopropylsulfonic acid, PDDA and PSS followed by the immobilization of MWCNT dispersed with PDDA made possible the sensitive and selective detection of glucose [117]. Schmidtke's group [118] described the construction of glucose biosensors by electrostatic L-b-L self-assembling of the positively charged poly[(vinylpyridine)Os(bipyridyl)₂Cl^{2+/3+}] and GOx containing SWCNT on a gold electrode functionalized with 11-mercaptopundecanoic acid. Rubianes and Rivas [45] reported

the use of the PEI-MWCNT immobilized at GCE as a platform for building a supramolecular architecture for glucose biosensing through the assembling of the negatively charged GOx. Chu et al. [119] proposed the adsorption of GOx at the gold NP modified CNT electrode. CNT were covalently immobilized on gold electrode through carbodiimide activation and linkage between carboxylic groups of CNT and amine residues of cysteamine. The resulting electrode was covered with a thin layer of Nafion to improve the stability and selectivity of the biosensor.

Wallace and co-workers [120] developed a new material consisting of an aligned, highly oriented carbon nanotubes array in three dimensions coated by a layer of polypyrrole, which allowed the immobilization of GOx onto the nanotubes array during pyrrole polymerization. A fast and sensitive response was also observed for a biosensor obtained by the immobilization of MWCNTs vertically adhered on a gold film followed by the adsorption of GOx [121]. The MWCNT-based biosensor exhibited a fast and sensitive response. The use of opened tubes allowed the accumulation of higher amounts of GOx and as a consequence of that, the stability of the biosensor largely increased.

Direct charge transfer between proteins and electrode surfaces is one of the important challenges of bioelectrochemists. In this sense, several contributions have demonstrated the direct electron transfer between the active center of GOx and the electrode modified with CNT. Dong and co-workers [122] have reported the electron transfer of GOx entrapped within the composite CNT/chitosan and attributed the improvement in the electron transfer to a better accessibility of the active site of GOx by a possible conformational change of GOx within the microenvironment. The direct electron transfer of GOx promoted by CNT dispersed in a solution of the cationic surfactant cetyltrimethylammonium bromide was also described [123]. The direct electron transfer of GOx at -0.45 V was observed at electrodes modified with a dispersion of CNTs in aminopropyltriethoxysilane APTES [124]. Dong and co-workers [125] proposed the quantification of glucose from the direct electron

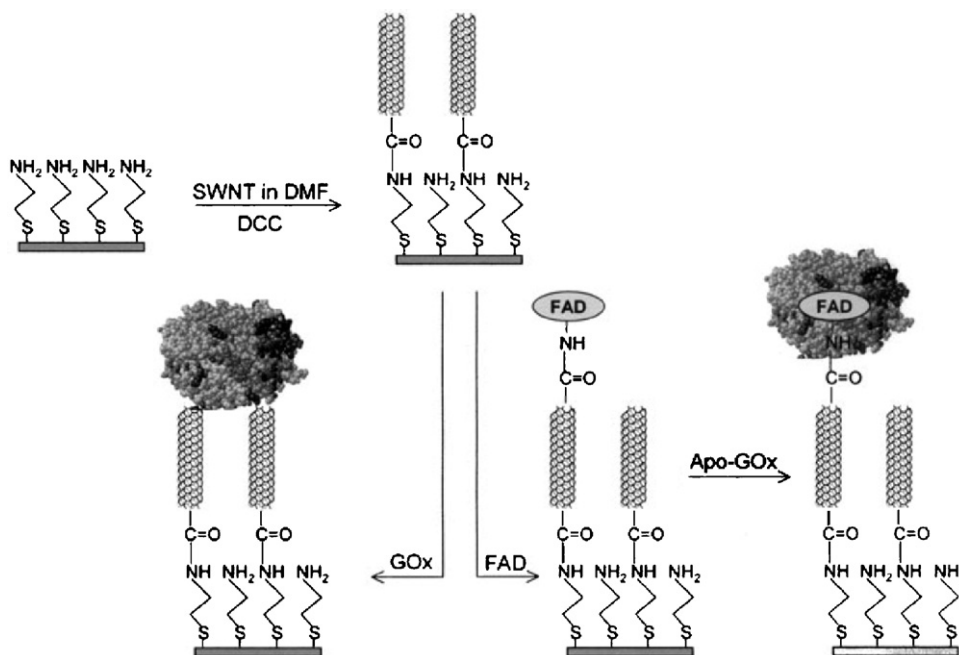


Fig. 6. Scheme of the protocol for the modification of a self-assembled monolayer modified gold electrode with aligned SWCNT and further modification with GOx or FAD and then apo-GOx. Adapted from reference [116] Gooding et al., Achieving Direct Electrical Connection to Glucose Oxidase Using Aligned Single Wall Carbon Nanotubes Array, *Electroanalysis* 17 (2005) pp. 38–46. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

transfer of GOx by using GOx immobilized on a GCE modified with CNTs dispersed in an ionic liquid.

The adsorption of GOx on bamboo-shaped CNT and the improvement in the electron transfer of GOx with the electrode through the N doping of CNT was also proposed [126].

Tsai et al. [127] prepared a nanobiocomposite film consisted of polypyrrole-functionalized MWCNT and GOx obtained by electrooxidation of pyrrole in aqueous solution containing appropriate amounts of functionalized CNT, incorporated within the net as contraion, and GOx. Wang and Musameh [128] proposed the highly sensitive biosensing of glucose by using anionic CNT as dopant in the preparation of conducting-polymer enzyme electrodes.

The low-potential amperometric biosensing of glucose based on the covalent attachment of GOx on platforms obtained by magnetic loading of CNT/nano-Fe₂O₃/chitosan composite on electrodes was also described [129]. A highly sensitive and selective glucose biosensor was obtained from the hydrogen peroxide oxidation at a GCE modified by immobilization of well-aligned MWCNTs, followed by the exposure to iron phthalocyanine and the incorporation of GOx during the electropolymerization of *o*-aminophenol [130].

The use of an enzyme alternative to GOx was also described. Glucose dehydrogenase (GDH) was attached to CNT-CHIT films by covalent bond through the use of glutaraldehyde (GDI) to cross-link the enzyme [131]. A very sensitive and selective determination of glucose was obtained from the facilitated oxidation of NADH in the presence of CNT, with successful application for the determination of glucose in urine.

4.1.2.2. Fructose biosensor. Magno and co-workers [62] reported an amperometric biosensor for fructose using CNTPE

covered by a polymer obtained from the electropolymerization of dihydroxybenzaldehyde. The fructose dehydrogenase was immobilized on different membranes placed on the top of CNTPEs and then covered with an additional polycarbonate membrane (0.03 μm pore size) to prevent fouling and microbial attack.

4.1.2.3. Cholesterol. A sensitive cholesterol biosensor was obtained by self-assembling of the positively charged Pt-CNT-CHIT and negatively charged PSS on gold electrodes [132]. The enzyme cholesterol oxidase was immobilized using glutaric dialdehyde. Shen's group also proposed the sensitive determination of cholesterol from the hydrogen peroxide reduction at -0.2 V by using a GCE modified with covalently attached histidine-MWCNT complex/nickel hexacyanoferrate nanoparticles and cholesterol oxidase. A biosensor consisting of the modification of a SPE with cholesterol esterase, cholesterol oxidase, peroxidase, potassium ferrocyanide and MWCNT was used for the determination of total cholesterol in blood with very good correlation with the clinical assays [133]. Tuzhi and co-workers [134] proposed the abrasive immobilization of a CNT and Pt composite obtained by chemical reduction of Pt salt and deposition on the top of the electrode previously modified with a dispersion of CNTs in a sol-gel solution. An amperometric biosensor based on the immobilization of an organic/inorganic hybrid material constituted by MWCNT/sol-gel chitosan/SiO₂ mixed with cholesterol oxidase and MWCNT dispersed in water was also described [135].

4.1.2.4. Lactate biosensor. Rubianes and Rivas [82] proposed the immobilization of lactate oxidase within CNTPE. The increase in the sensitivity towards lactate through the catalytic

oxidation of NADH at GCE modified with SWCNT and lactate dehydrogenase film was also proposed [136]. A very original work showed for the first time a method for an enzymatically catalytic polymerization to prepare polypyrrol–lactate oxidase–CNT or polypyrrol–lactate oxidase composite using lactate oxidase to initiate the polymerization of pyrrole in the presence and absence of CNT [137].

4.1.2.5. Phenols and catechols biosensor. A highly sensitive biosensor for phenols and catechols was obtained by incorporation of polyphenol oxidase within CNTPE. The response for dopamine was 12-fold more sensitive than at CPE–PPO [82].

4.1.2.6. Hydrogen peroxide biosensor. Zhao et al. [138] proposed the catalytic reduction of hydrogen peroxide at an electrode obtained by immobilization of myoglobin on MWCNT-modified GCE with a voltammetric response stable after 1 month at 4 °C or 1 week stored in air. The electrochemical behavior of myoglobin on acid-treated-MWCNT–GCE and the potential application for the development of a nitric oxide biosensor was also proposed [139]. Zhao et al. [140] described the direct electron transfer between the strongly adsorbed cytochrome *c* and MWCNTs–GCE and the detection of hydrogen peroxide.

Shi and co-workers [141] studied the catalytic activity of MWCNTs–horseradish peroxidase (HRP)–GCE towards hydrogen peroxide. The biosensor was prepared by dropping HRP and bovin seric albumin on the MWCNTs–GCE followed by the cross-linking with glutaraldehyde. Glucose and lactate can be also detected through the additional immobilization of the corresponding enzymes from the hydrogen peroxide enzymatically generated.

The covalent attachment of myoglobin and HRP onto the ends of vertically oriented shortened SWCNTs through covalent attachment between the carboxyl groups of nanotubes and lysine residues of the proteins made possible the detection of 70 and 50 nM for hydrogen peroxide, at Mb- and HRP-modified electrodes, respectively [142]. Qian and Yang [143] proposed a hydrogen peroxide biosensor obtained by cross-linking of HRP on a MWCNT–CHIT composite film modified GCE. Qian and Yang [143] reported the sensitive and selective determination of hydrogen peroxide based on the use of a GCE modified with a MWCNT/chitosan dispersion cross-linked with horseradish peroxidase by glutaraldehyde. Fabregas and co-workers [66] showed the advantages of a MWCNT/polysulfone biocomposite membrane modified SPE in the detection of hydrogen peroxide. HRP was incorporated by phase inversion technique and the resulting electrode was useful for the detection of hydrogen peroxide. Hu and co-workers [144] reported the adsorption of the positively charged myoglobin or hemoglobin on the surface of negatively charged oxidized MWCNT and further immobilization on pyrolytic graphite. The proteins catalyze the reduction of oxygen and hydrogen peroxide. Yuan and co-workers [145] proposed a similar strategy through the immobilization of previously oxidized MWCNT on GCE, followed by the assembling of the positively charged hemoglobin and negatively charged gold nanoparticles. The authors demonstrated the

direct electron transfer of hemoglobin (Hb) on Hb/gold nanoparticles/Hb/GNP/Hb/MWCNT/GCE.

A chemically etched 100 μm diameter Pt microelectrode was grown on a flat plate with CNTs mixed with hemoglobin and the resulting electrode allowed the detection of hydrogen peroxide at -0.80 V through the catalytic activity of the immobilized hemoglobin [146]. Another strategy reported by Lin and co-workers [147] was the development of a novel nanobiocomposite material through the immobilization of MWCNT, Nafion and heme proteins on a choline-modified GCE. The immobilized proteins present activity of peroxidase working on the electrocatalytic reduction of oxygen, hydrogen peroxide, nitric oxide, among others. Chen and Lu [148] proposed a hydrogen peroxide biosensor through the encapsulation of hemoglobin in a composite film of previously oxidized CNT. A hydrogen peroxide biosensor was obtained by entrapping HRP in a sol–gel matrix organically modified (ormosil) doped with ferrocene monocarboxylic acid-bovin serum albumin conjugate and MWCNT [149]. Smyth and co-workers proposed [150] a biosensor based on the immobilization of HRP onto electropolymerized polyaniline films doped with CNTs. They found an enhanced stability and sensitivity towards hydrogen peroxide compared to the biosensor without CNT. Dong's group [151–153] reported the direct electron transfer of 11-microperoxidase on electrodes containing MWCNT immobilized following different strategies, indicating that CNT can wire the protein to the electrode, catalyzing the reduction of hydrogen peroxide and oxygen.

4.1.2.7. Alcohol biosensor. A composite biosensor was prepared by adding the desired amount of alcohol dehydrogenase (ADH) and NAD^+ cofactor to a 50/50% (w/w) MWCNT/Teflon composite [54]. The marked decrease in the overvoltage for the oxidation of the liberated NADH allowed the efficient determination of alcohols. Rubianes and Rivas [82] also proposed the immobilization of ADH (12.0%, w/w) into a CNTPE in the presence of NAD^+ (12.0%, w/w). Based on the excellent electrocatalytic properties of MWCNTs towards the oxidation of NADH, a very fast and sensitive response for ethanol was obtained at CNTPE–ADH– NAD^+ . Liu and Cai [154] proposed the self-assembling of alcohol dehydrogenase onto the surface of GCE modified with SWCNT wrapped by the polycation PDDA, followed by the immobilization of a layer of Nafion. The biosensor allowed the sensitive detection of ethanol, with detection limits of 90 μM .

4.1.2.8. Choline and related compounds. Zhang and co-workers [155] proposed an amperometric sensor for acetylthiocholine by binding the enzyme acetylcholinesterase on a MWCNT-cross-linked chitosan composite. The detection limit for acetylthiocholine was 0.10 mM. Chen and co-workers [156] proposed an amperometric biosensor for the detection of choline by immobilization of choline oxidase into a sol–gel silicate film on MWCNT-modified platinum electrode, being the detection limit $1 \times 10^{-7}\text{ M}$.

4.1.2.9. Organophosphate pesticides. Liu and Lin [157] proposed a biosensor for flow injection amperometric detection

of organophosphate pesticides and nerve agents based on the immobilization of acetylcholinesterase by self-assembling on PDDA/CNT-modified GCE. The electrocatalytic activity of CNT made possible a significant improvement in the electrochemical detection of the enzymatic product, thiocholine, at 150 mV. Under the optimal conditions, after 6 min inhibition time, it was possible to detect 0.4 pM of paraoxon.

Wang's group presented several works for the quantification of organophosphorous compounds [158–160]. In one of the works [158] they proposed an amperometric biosensor for the quantification of organophosphorous pesticides using a CNT/Nafion/GCE modified with organophosphorous hydrolase from the improved electrochemical detection of the enzymatically generated *p*-nitrophenol. The other two works are related to the detection of organophosphorous compounds from the enzymatic inhibition. In one case [159] they reported the use of MWCNT/DMF/SPE modified with acetylcholinesterase and the determination of paraoxon from the inhibition of the thiocholine formation. The other alternative proposed by this group was the use of a combination of two enzymes: acetyl cholinesterase and choline oxidase covalently attached to MWCNT/DMF/SPE [160]. In this way, the detection can be performed from the inhibition of the hydrogen peroxide generation in the presence of parathion after the addition of acetylcholine, conversion into choline by one of the enzymes and final oxidation by choline oxidase.

4.2. Carbon nanotubes-based-DNA biosensors

In these biosensors the biorecognition layer is a DNA molecule. There are, basically, two types of DNA biosensors, for the detection of the hybridization event and for the detection of the DNA–drugs interaction or DNA damage.

There are two fundamental aspects in the development of hybridization biosensors, sensitivity and selectivity. Traditional methods for detecting the hybridization event are too slow and require special preparation. Therefore, there is a great interest for developing new hybridization biosensors, and the electrochemical ones represent a very interesting alternative. An electrochemical DNA hybridization biosensor basically consists of an electrode modified with a single stranded DNA (or PNA) called probe [161]. The immobilization of DNA probe on the electrode is the first and most critical step in the preparation of the biosensor. The second step is the hybrid formation under selected conditions of pH, temperature and ionic strength, and the last one, involves the detection of the double helix formation by a given methodology that allows to obtain an electrical signal that clearly demonstrates that the sequence-specific biorecognition event has taken place.

Another interesting aspect of sensors containing DNA as biorecognition layer is the detection of chemical and physical damage produced on DNA. In this case, it is necessary to immobilize preferentially double stranded DNA at the electrode surface to build the probe. The second step consists of the interaction of the DNA layer confined to the electrode with the given damage agent under controlled conditions, and the last step is the transduction of the signal, either from the oxi-

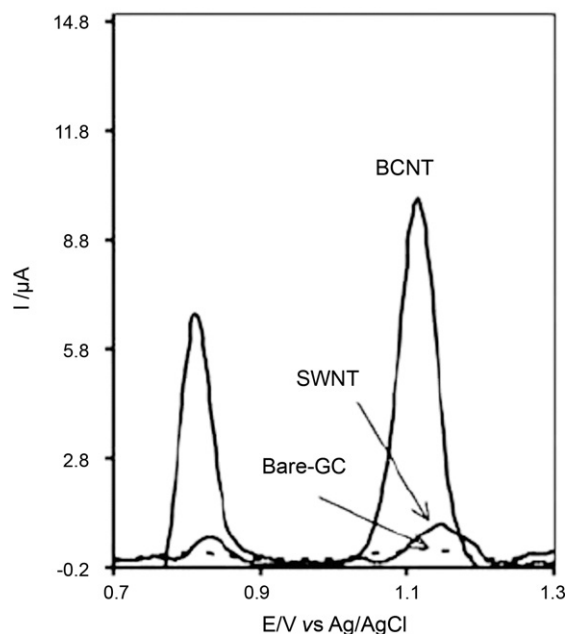


Fig. 7. Differential pulse voltammograms obtained at bare GCE, SWCNT-modified GCE and bamboo CNT-modified GCE. DNA concentration 0.4 mg/mL. Adapted from reference [165].

dation/reduction of the nucleobases, damage agent, and/or the corresponding adducts [161].

Pedano and Rivas [162] reported the adsorption (mainly hydrophobic) and electrooxidation of oligo and polynucleotides at a CNTPE. No shifting in the peak potential was obtained, indicating that the main effect of CNTs is on the adsorption of the nucleic acids. A pretreatment of 1.3 V for 20 s in a 0.200 M acetate buffer solution pH 5.0 demonstrated to be the most effective for the adsorption and oxidation of nucleic acids. Ye and Ju [163] reported the use of SPE modified with MWCNT for the fast and sensitive detection of DNA and RNA from the electrooxidation of guanine and adenine residues catalyzed by MWCNT. The use of SPE modified with nanostructured films of MWCNT, hydroxyapatite and montmorillonite for developing DNA biosensors was reported by Labuda and co-workers [164]. They evaluated the presence of the nucleic acid from the redox signals of the marker $[\text{Co}(\text{phen})_3]^{3+}$ and guanine residues. In an interesting work, Gooding and co-workers reported the advantages of using bamboo type carbon nanotubes over single stranded CNT on the oxidation of DNA bases [165]. The enhancement in the oxidation signals of guanine residues was attributed to the presence of edge planes of graphene at regular intervals along the walls of the bamboo CNT. Fig. 7 clearly shows the noticeable enhancement in the voltammetric detection of DNA at bamboo CNT-based electrode. Bollo et al. [44] proposed the use of GCE modified with oxidized MWCNT dispersed in chitosan for the quantification of DNA from the oxidation signal of the intrinsic DNA residues.

Wang et al. [166] reported a significantly higher sensitivity for DNA oxidation at CNTs–GCE compared to GCE, although the oxidation occurs at more elevated potentials, indicating that CNTs promotes the interfacial accumulation more than the elec-

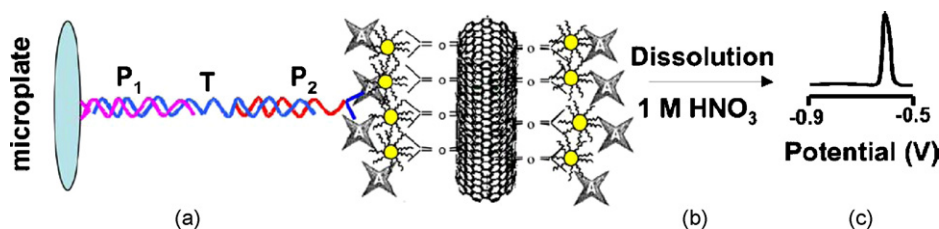


Fig. 8. Schematic representation of the protocol for the biosensor: (a) dual hybridization event for the sandwich hybridization assay, leading to the capture of the CdS-loaded CNT tags in the microwell; (b) dissolution of CdS tracer; (c) stripping voltammetric detection of cadmium at a mercury-coated GCE. P1, DNA probe 1; T, DNA target; P2, DNA probe 2. From reference [168].

tron transfer. A guanine oxidation signal 17-fold higher than that at the bare GCE was obtained after a short period of accumulation (3 min). The CNT–GCE was also used as a detector for the hybridization event using the advantageous two-surface hybridization scheme. The presence of CNTs allowed an efficient way to amplify the label-free electrochemical detection of DNA hybridization. Performing the digestion of the DNA sample in the presence of copper, well defined hybridization signals were obtained for BRCA1 breast cancer gene after 20 min hybridization, with detection limit of 40 ng/mL.

Wang et al. [167] described the use of CNT in two directions, for the recognition and for the transduction event. For the biorecognition event, alkaline phosphatase was bound to CNTs through a carbodiimide linker. Once the hybrids were formed, the alkaline phosphatase bound to CNTs catalyzes the formation of the electroactive enzymatic product α -naphthol and the sensitivity improved almost 10^4 times. When CNTs were also present as modifier of the GCE, a second amplification was obtained (30 fold), in connection with the accumulation of the enzymatic product α -naphthol in the presence of nanotubes. A detection limit of 1 fg/mL was obtained after 20 min accumulation. Wang et al. [168] also proposed an attractive alternative for the amplification of DNA hybridization based on the ultrasensitive stripping-voltammetric detection of the dissolved CdS tags coming from the SWCNTs at a mercury film-glassy carbon electrode in connection with the square wave voltammetric detection of cadmium (Fig. 8). Under these conditions the detection limit at -0.60 V was 40 pg/mL.

He and Dai [169] proposed a new avenue for detecting the hybridization event by using aligned CNT–DNA probes immobilized on the tip and wall of plasma aligned CNTs. The CNTs were oxidized in acetic acid–plasma, and then the DNA probe was grafted through the carboxyl functions of CNT and the amino terminal group of the DNA located at 5'-phosphate position in the presence of EDC. The detection of the hybridization event was performed through the amperometric response of ferrocenecarboxaldehyde used as a label of the target DNA.

Fang and co-workers [170] described a sensing layer for the detection of the hybridization event obtained by immobilization of oxidized MWCNTs/DMF on GCE, followed by the incorporation of DNA within the polypyrrole film during the electropolymerization process. The detection limit at 42°C was 1.0×10^{-8} M. Fang and co-workers [171] also proposed a DNA biosensor based on the covalent attachment of the 5'-amino probe sequence on an oxidized MWCNT/GCE. The changes in

the voltammetric signal of daunomycin were used as hybridization indicator. In another work, Fang and co-workers showed the advantages of an electrochemical DNA biosensor based on the use of a dispersion of Pt–NP–MWCNT/Nafion/GCE and daunomycin as redox indicator [172]. Fang and co-workers [173] proposed a biosensor for the detection of a portion of the human porphobilinogen deaminase PBGD promoter by using a GCE modified with polypyrrole/carboxylated MWCNT containing the 5'(NH₂) probe attached to the mercaptoacetic-coated magnetic nanoparticles. A detection limit of 2.3×10^{-11} M was obtained from the decrease in the reduction peak current of daunomycin.

The combination of ZrO₂ nanoparticles with MWCNT dispersed in chitosan allowed the fabrication of successful DNA hybridization biosensors [174]. The probe sequence was immobilized by adsorption on the glassy carbon electrode modified with the dispersion and the detection was performed from the improved redox behavior of daunomycin, through the synergistic effects of nano-porous ZrO₂ and MWCNT.

In a very original work, the use of the interaction between a single stranded DNA binding protein and single stranded DNA was proposed for the first time for detecting the hybridization event [175]. As a consequence of the interaction, the voltammetric signal for the nucleic acid (oxidation of guanine residues) decreases while the one for the protein (oxidation of tyrosine and tryptophan) increases. When the double helix is formed, the guanine oxidation signal is still observed, while the one for the oxidation of protein residues disappears.

Wang and co-workers [176] reported a new methodology for the electrochemical detection of DNA or proteins based on the use of enzyme multilayers on CNT for monitoring sandwich hybridization and antibody–antigen interactions associated with alkaline phosphatase markers. Under these conditions, it was possible the detection of 80 DNA copies and 2000 proteins molecules. Yao and co-workers [177] reported the electrostatic assembly of calf thymus DNA on oxidized-MWCNTs-modified gold electrode using PDDA as linker. The nanotubes films were prepared by dropping the suspension of MWCNTs in water (0.1 mg/mL) and evaporating under vacuum at 50°C . The authors used the electrode for detecting the interaction of DNA with the intercalator chlorpromazine. He and Bayachou [178] proposed the immobilization of DNA on modified SWCNT by the L-b-L technique using PDDA. The resulting electrode was proposed to study the DNA damage by nitric oxide. Yao and co-workers [179] described a DNA biosensor based on the use of CNT dispersed in chitosan. The authors proposed the indi-

rect detection of sperm DNA through the use of the intercalator methylene blue. Another alternative to immobilize calf-thymus DNA on multi-walled CNTs activated with EDC and NHS was obtained by immersion in a phosphate buffer solution containing 2 mg/mL of ssDNA for several hours [30]. The electrode was used to study the interaction with ethidium bromide (EB).

4.3. Immunosensing schemes

Immunosensors are based on the high affinity reactions antigen/antibody. Several strategies can be used to immobilize the recognition element, either the antibody or the antigen, depending on the selected scheme. The detection of the recognition event uses the same principle as the enzymatic immunoassay. In general, an enzyme is coupled to the recognition layer (the antigen or antibody) and the enzymatic reaction is developed once the antigen/antibody interaction occurred and after the addition of the substrate and electrochemically detection of the product [180].

An amperometric biosensor based on the adsorption of antibodies onto perpendicularly oriented assemblies of single-wall CNTs called forest was proposed [181]. The immobilization of the anti-biotin antibody was performed by incubation for 3 h on the surface of SWCNTs. After adequate washing, the electrode was blocked with 2% bovine serum albumine in PBS. The detection of HRP bound to biotin was evaluated at -0.3 V with 1 mM hydroquinone and 400 μ M hydrogen peroxide. The detection limit was 2.5 nM and the linear range was up to 25 nM. Unlabelled biotin was detected in a competitive approach with a detection limit of 16 μ M.

Bianco and co-workers [182] presented the functionalization of CNTs with two polypeptides employing two different strategies. In both cases, the CNTs were previously modified to expose amine groups. In the first methodology, a model peptide was condensated on the CNTs surface through a linker in DMF, while in the second, the peptide was the B-cell epitope from the foot and mouse disease virus (FMVD) immobilized via succinimide. Immunological assays coupled to surface plasmon resonance showed that the adsorbed peptide was recognized by its polyclonal antibodies indicating that the adsorption process did not produce significant conformational changes in the secondary structure of the peptide.

Maehashi et al. [183] proposed a label-free electrochemical immunosensor based on the use of microelectrode arrays modified with SWCNT containing the antibody anti-total prostate-specific antigen and the voltammetric detection of the total prostate-specific antigen from the oxidation of tyrosine and tryptophan, with very good detection limits (0.25 ng/mL). Another strategy was the development of an amperometric immunoassay electrode through the use of vertically aligned arrays of SWCNT on pyrolytic graphite bound to antibodies through the carboxylated ends [184]. The authors proposed two alternatives for detection of albumin, unmediated immunosensing using hydrogen peroxide labels, or using electron mediators to improve the sensitivity. Schultz and co-workers [185] proposed a label-free immunosensor based on the use of a CNT array electrode grown on a Fe/Al₂O₃/SiO₂/Si substrate, then

cast in epoxy, polished, and activated to expose carboxylic groups. The resulting electrode was used to covalently attach anti-mouse IgG. The biorecognition process with mouse IgG was evaluated from cyclic voltammetry and electrochemical spectroscopy impedance. Under these conditions the detection limit was 200 ng/mL. Fábregas and co-workers [186] described a very original electrochemical biosensor based on the encapsulation of MWCNT and rabbit IgG within a polysulfone membrane on disposable SPE. Direct and competitive immunoassays were performed in the presence of anti-rabbit IgG–HRP conjugate after the addition of hydrogen peroxide and hydroquinone as mediator. The efficiency of the new sensor arises from the combination of the conducting properties of MWCNT with the biocompatibility and flexibility of the polysulfone polymer and screen printed technology.

5. Final considerations

CNTs have demonstrated to be an excellent material for the development of electrochemical sensors. CNTs pretreatments, CNT-based electrodes preparation and the selection of the biomolecule immobilization procedure depend on the system under investigation. The incorporation of CNTs within composites offers the advantages of an easy and fast preparation, while the dispersion of CNTs in polyelectrolytes represents a very convenient alternative as a platform for further design of different (bio)sensors once immobilized at the electrode.

The combination of the unique properties of CNTs with the powerful recognition properties of biomolecules and the known advantages of the electrochemical techniques represents a very good alternative for the development of (bio)sensors able to address future biosensing challenges in clinical diagnostics, environmental monitoring and security control, among others.

Congratulations



When I arrived in Las Cruces in 1995 I found an excellent scientist with an incredible energy to work and create new ideas, that opened the laboratory very early every day and celebrated the paper 500 with the same happiness as the paper number 100 ... When I left, I had the unique opportunity to take with me, not only an incredible scientific baggage that enormously helped me in my academic career, but also an excellent FRIEND, Joe Wang ...

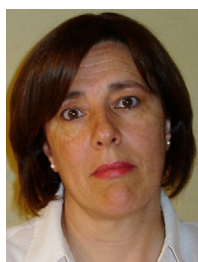
I just can say ... Dr Wang ... MANY MANY THANKS for your help and for your friendship!!! ... HAPPY BIRTHDAY!!!!!!!

Gustavo



I would like to express my congratulations to an excellent scientist and a wonderful person as Professor Joseph Wang. Every moment in the Lab Sensor at NMSU and ASU during 2004–2005 will be unforgettable for me. His quality as a person and his amazing ideas make him an exceptional scientist. I am profoundly thankful for everything I have learnt by his side. Many Congratulations and Happy 60th Birthday Professor Wang!

Marcela.



Goethe said: If I could give an account of all that I owe to my great predecessors and contemporaries, there would be but a small balance in my favour. In my particular case, Professor Wang, would be mainly responsible for such a small balance.

Concha.

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References

- [1] S. Iijima, *Nature* 354 (2001) 56.
- [2] H. Dai, *Acc. Chem. Res.* 35 (2002) 1035.
- [3] Q. Zhao, Z. Gan, Q. Zhuang, *Electroanalysis* 14 (23) (2002), and references therein.
- [4] M.L. Cohen, *Mater. Sci. Eng. C* 15 (2001) 1.
- [5] R.H. Baughman, A. Zakhidov, W.A. de Heer, *Science* 297 (2002) 787.
- [6] P.M. Ajayan, *Chem. Rev.* 99 (1999) 1787.
- [7] P.M. Ajayan, O.Z. Zhou, in: G. Dresselhaus, Ph. Avouris (Eds.), *Applications of Carbon Nanotubes in Carbon Nanotubes*, Springer, Heidelberg, 2001, pp. 391–425.
- [8] P.M. Ajayan, T.W. Ebbesen, *Rep. Prog. Phys.* 60 (1997) 1025.
- [9] Carbon Nanotubes, Physics web, 1998.
- [10] M.S. Dresselhaus, Y.M. Lin, O. Rabin, A. Jorio, A.G. Souza Filho, M.A. Pimenta, R. Saito, G. Ge, G. Samsonidze, G. Dresselhaus, *Mater. Sci. Eng. C* 23 (2003) 129.
- [11] K. Balasubramanian, M. Burghard, *Small* 1 (2005) 180.
- [12] C.T. Kingston, B. Simard, *Anal. Lett.* 15 (2003) 3119.
- [13] O. Zhou, H. Shimoda, B. Gao, S. Oh, L. Fleming, G. Yue, *Acc. Chem. Res.* 35 (2002) 1045.
- [14] H.H. iura, T.W. Ebbe sen, K. Tanigaki, *Adv. Mater.* 7 (1995) 275.
- [15] P.J. Britto, K.S.V. Santhanam, V. Alonso, A. Rubio, P.M. Ajayan, *Adv. Mater.* 11 (1999) 11.
- [16] C.E. Banks, T.J. Davies, G.G. Wildgoose, R.G. Compton, *Chem. Commun.* (2005) 829;
C.E. Banks, R.G. Compton, *Analyst* 130 (2005) 1232;
C.E. Banks, R.G. Compton, *Analyst* 131 (2006) 15.
- [17] A. Merkoci, *Microchim. Acta* 152 (2006) 157.
- [18] C.E. Banks, A. Croosley, C. Salter, S.J. Wilkins, R.G. Compton, *Angew. Chem. Int. Ed.* 45 (2006) 2533.
- [19] J.J. Gooding, *Electrochim. Acta* 50 (2005) 3049.
- [20] E. Katz, I. Willner, *Chem. Phys. Chem.* 5 (2004) 1084.
- [21] A. Merkoci, M. Pumera, X. Llopis, B. Pérez, M. del Valle, S. Alegret, *Trends Anal. Chem.* 24 (2005) 826.
- [22] J. Wang, *Electroanalysis* 17 (2005) 7.
- [23] M. Valcárcel, B.M. Simonet, S. Cárdenas, B. Suárez, *Anal. Bioanal. Chem.* 382 (2005) 1783.
- [24] M. Jiang, Y. Lin, in: G.A. Grimes (Ed.), *Encyclopedia of Sensors*, vol. 2, American Scientific Publisher, 2006, ISBN 1-58883-058-6, pp. 25–51.
- [25] F. Valentini, A. Amine, S. Orlanducci, M.L. Terranova, G. Palleschi, *Anal. Chem.* 75 (2003) 5413.
- [26] S. Sotiropoulou, N.A. Chaniotakis, *Anal. Bioanal. Chem.* 375 (2003) 103.
- [27] M. Musameh, J. Wang, A. Merkoci, Y. Lin, *Electrochem. Commun.* 4 (2002) 743.
- [28] A. Salimi, C.E. Banks, R.G. Compton, *Analyst* (2004) 225.
- [29] K. Gong, Y. Dong, S. Xiong, Y. Chen, L. Mao, *Biosens. Bioelectron.* 20 (2004) 253.
- [30] M. Guo, J. Chen, D. Liu, L. Nie, S. Yao, *Bioelectrochemistry* 62 (2004) 29.
- [31] C.G. Hu, W.L. Wang, S.X. Wang, W. Zhu, Y. Li, *Diamond Relat. Mater.* 12 (2003) 1295.
- [32] C.G. Hu, W.L. Wang, K.J. Liao, G.B. Liu, Y.T. Wang, *J. Phys. Chem. Solids* 65 (2004) 1731.
- [33] Z.H. Wang, Q.-L. Liang, Y.-M. Wang, G.-A. Luo, *J. Electroanal. Chem.* 540 (2003) 129.
- [34] M. Pumera, *Langmuir* 23 (2007) 6453.
- [35] F. Valentini, S. Orlanducci, M.L. Terranova, A. Amine, G. Palleschi, *Sens. Actuators B* 100 (2004) 117.
- [36] R. Antiochia, I. Lavagnini, F. Magno, F. Valentini, G. Palleschi, *Electroanalysis* 16 (2004) 1451.
- [37] J. Wang, A.-N. Kawde, M. Musameh, *Analyst* 128 (2003) 912.
- [38] J. Wang, M. Li, Z. Shi, N. Li, Z. Gu, *Anal. Chem.* 74 (2002) 1993.
- [39] J. Wang, M. Li, Z. Shi, N. Li, Z. Gu, *Electrochim. Acta* 47 (2001) 651.
- [40] X.X. Yan, D.-W. Pang, Z.-X. Lu, J.-Q. Li, H. Tong, *J. Electroanal. Chem.* 1 (2004) 47.
- [41] J. Wang, M. Musameh, Y. Lin, *J. Am. Chem. Soc.* 125 (2003) 2408.
- [42] G. Rivas, S. Miscoria, J. Desbrieres, G. Barrera, *Talanta* 71 (2007) 270.
- [43] Y. Liu, X. Qu, H. Guo, H. Chen, B. Liu, S. Dong, *Biosens. Bioelectron.* 21 (2006) 2195.
- [44] S. Bollo, N. Ferreyra, G. Rivas, *Electroanalysis* 19 (2007) 833.
- [45] M.D. Rubianes, G.A. Rivas, *Electrochem. Commun.* 9 (2007) 480.
- [46] A. Sánchez Arribas, E. Bermejo, M. Chicharro, A. Zapardiel, G.L. Luque, N.F. Ferreyra, G.A. Rivas, *Anal. Chim. Acta* 596 (2007) 183.
- [47] Y. Jalit, M.C. Rodríguez, G.A. Rivas, unpublished results.
- [48] J. Tkac, T. Ruzgas, *Electrochem. Commun.* 8 (2006) 899.
- [49] K. Wu, Y. Sun, S. Hu, *Sens. Actuators B* 96 (2003) 658.
- [50] J. Zhang, L. Gao, *Mater. Lett.* 61 (2007) 3571.
- [51] Z. Wang, S. Xiao, Y. Chen, *J. Electroanal. Chem.* 589 (2006) 237.
- [52] R.R. Moore, C.E. Banks, R. Compton, *Anal. Chem.* 76 (2004) 2677.
- [53] J. Wang, M. Musameh, *Anal. Chem.* 75 (2003) 2075.
- [54] J. Wang, M. Musameh, *Anal. Lett.* 36 (2003) 2041.
- [55] J.J. Davis, R.J. Coles, H.A.O. Hill, *J. Electroanal. Chem.* 440 (1997) 279.
- [56] P.J. Britto, K.S.V. Santhanam, P.M. Ajayan, *Bioelectrochem. Bioenergetics* 41 (1996) 121.
- [57] M.D. Rubianes, G.A. Rivas, *Electrochem. Commun.* 5 (2003) 689.

- [58] G.A. Rivas, M.D. Rubianes, M.L. Pedano, N.F. Ferreyra, G.L. Luque, M.C. Rodríguez, S.A. Miscoria, *Electroanalysis* 19 (2007) 823.
- [59] M. Chicharro, A. Sanchez, E. Bermejo, A. Zapardiel, M.D. Rubianes, G.A. Rivas, *Anal. Chim. Acta* 543 (2005) 84.
- [60] M. Chicharro, E. Bermejo, M. Moreno, A. Sanchez, A. Zapardiel, G. Rivas, *Electroanalysis* 17 (2005) 476.
- [61] N.S. Lawrence, R.P. Deo, J. Wang, *Talanta* 63 (2004) 443.
- [62] R. Antiochia, I. Lavagnini, F. Magno, *Anal. Lett.* 37 (2004) 1657.
- [63] J. Wang, G. Chen, M. Wang, M.P. Chatrathi, *Analyst* 129 (2004) 512.
- [64] A. Sánchez Arribas, M. Chicharro, E. Bermejo, A. Zapardiel, G.L. Luque, N.F. Ferreyra, G.A. Rivas, *Anal. Chim. Acta* 577 (2006) 183.
- [65] G.L. Luque, N.F. Ferreyra, G.A. Rivas, *Microchim. Acta* 152 (2006) 277.
- [66] S. Sánchez, M. Pumera, E. Cabruja, E. Fabregas, *Analyst* 132 (2007) 142.
- [67] J. Wang, M. Musameh, *Analyst* 129 (2004) 1.
- [68] X.P. Diao, Z. Liu, B. Wu, X. Nan, J. Zhang, Z. Wei, *Chem. Phys. Chem.* 10 (2002) 898.
- [69] C.V. Nguyen, L. Delzeit, A.M. Carsell, J. Li, J. Han, M. Meyyappan, *NanoLetters* 2 (2002) 1079.
- [70] A.B. Artyukhin, O. Bakajin, P. Stroeve, A. Noy, *Langmuir* 20 (2004) 1442.
- [71] L. Liu, T. Wang, J. Li, Z.-X. Guo, L. Dai, D. Zhang, D. Zhu, *Chem. Phys. Lett.* 367 (2003) 747.
- [72] M.C. Rodríguez, M.D. Rubianes, G.A. Rivas, unpublished results.
- [73] H. Boo, R.-A. Jeong, S. Park, K.S. Kim, K.H. An, Y.H. Lee, J.H. Han, H.C. Kim, T.D. Chung, *Anal. Chem.* 78 (2006) 617.
- [74] H. Luo, Z. Shi, Z. Gu, Q.Z. Zhuang, *Anal. Chem.* 73 (2001) 915.
- [75] J. Wang, M. Li, Z. Shi, N. Li, Z. Gu, *Electroanalysis* 14 (2002) 225.
- [76] C. Hu, X. Chen, S. Hu, *J. Electroanal. Chem.* 586 (2006) 77.
- [77] Y. Sha, L. Qian, Y. Ma, H. Bai, X. Yang, *Talanta* 556 (2006) 74.
- [78] H.-S. Wang, T.-H. Li, W.-L. Jia, H.-Y. Xu, *Biosens. Bioelectron.* 22 (2006) 664.
- [79] L.-Q. Rong, C. Yang, Q.-Y. Qian, X.-H. Xia, *Talanta* 72 (2007) 819.
- [80] X. Kang, Z. Mai, X. Zou, P. Cai, J. Mo, *Anal. Biochem.* 369 (2007) 71.
- [81] S. Hrapovic, E. Majid, Y. Liu, K. Male, J.H.T. Luong, *Anal. Chem.* 78 (2006) 5504.
- [82] M.D. Rubianes, G.A. Rivas, *Electroanalysis* 17 (2005) 73.
- [83] M. Zhang, W. Gorski, *Anal. Chem.* 77 (2005) 3960.
- [84] J. Liu, S. Tian, W. Knoll, *Langmuir* 21 (2005) 5596.
- [85] Z. Xu, N. Gao, S. Dong, *Talanta* 68 (2006) 753.
- [86] R.P. Deo, N.S. Lawrence, J. Wang, *Analyst* 129 (2004) 1076.
- [87] J. Okuno, K. Maehashi, K. Matsumoto, K. Kerman, Y. Takamura, E. Tamiya, *Electrochem. Commun.* 9 (2007) 13.
- [88] J. Wang, M. Musameh, *Anal. Chim. Acta* 511 (2004) 33.
- [89] J. Wang, T. Tangkuaram, S. Loyprasert, T. Vázquez-Álvarez, W. Veerasi, P. Kanatharana, P. Thavarungkul, *Anal. Chim. Acta* 581 (2007) 1.
- [90] M.C. Rodríguez, J. Sandoval, L. Galicia, S. Gutiérrez-Granados, G.A. Rivas, unpublished results.
- [91] D. Vega, L. Agüí, A. González-Cortés, P. Yáñez-Sedeño, J.M. Pingarrón, *Talanta* 71 (2007) 1031.
- [92] C. Hu, C. Yang, S. Hu, *Electrochem. Commun.* 9 (2007) 128.
- [93] J. Cunningham, *Introduction to Bioanalytical Sensors*, J. Wiley & Sons, Inc., 1998, p. 86.
- [94] G. Ramsay, *Commercial Biosensors. Applications to Clinical, Bioprocess, and Environmental Samples*, John Wiley & Sons, Inc., NY, 1998.
- [95] Y. Lin, L.F. Allard, Y.-P. Sun, *J. Phys. Chem. B* 108 (2004) 3760.
- [96] B.R. Azamian, J.J. Davis, K.S. Coleman, C.B. Bagshaw, M.L.H. Green, *J. Am. Chem. Soc.* 124 (2002) 12664.
- [97] R.J. Chen, Y. Zhang, D. Wang, H. Dai, *J. Am. Chem. Soc.* 123 (2001) 3838.
- [98] J.J. Gooding, R. Wibons, J. Liu, W. Yang, D. Losic, S. Orbons, F.J. Mearns, J.G. Shapter, D.B. Hibbert, *J. Am. Chem. Soc.* 125 (2003) 9006.
- [99] B. Perez, M. Pumera, M. del Valle, A. Merkoci, S. Alegret, J. Nanosci. Nanotechnol. 5 (2005) 1694.
- [100] J. Wang, M. Musameh, *Analyst* 128 (2003) 1382.
- [101] N. Zhang, J. Xie, V.K. Varadam, *Smart Mater. Struct.* 15 (2006) 123.
- [102] M. Yang, Y. Yang, Y. Liu, G. Shen, R. Yu, *Biosens. Bioelectron.* 21 (2006) 1125.
- [103] S. Hrapovic, Y. Liu, K.B. Male, J.H.T. Luong, *Anal. Chem.* 76 (2004) 1083.
- [104] A. Salimi, R.G. Compton, R. Hallaj, *Anal. Biochem.* 333 (2004) 19.
- [105] H. Tang, J. Chem, S. Yao, L. Nie, G. Dong, Y. Kuang, *Anal. Biochem.* 331 (2004) 89.
- [106] J. Lim, J. Wei, J. Lin, Q. Li, J.K. You, *Biosens. Bioelectron.* 20 (2005) 2341.
- [107] H. Xue, W. Sun, B. He, Z. Sheu, *Synth. Met.* 135–136 (2003) 831.
- [108] Y.-C. Tsai, S.-C. Li, J.-M. Chen, *Langmuir* 21 (2005) 3653.
- [109] F. Qu, M. Yang, J. Chen, G. Shen, R. Yu, *Anal. Lett.* 39 (2006) 1785.
- [110] P.P. Joshi, S.A. Merchant, Y. Wang, D.W. Schmidtke, *Anal. Chem.* 77 (2005) 3183.
- [111] Y. Lin, F. Lu, Y. Tu, Z. Ren, *NanoLetters* 4 (2004) 191.
- [112] J. Li, Y.-B. Wang, J.-D. Q. D.-C. Sun, X.-H. Xia, *Anal. Bioanal. Chem.* 383 (2005) 918.
- [113] F. Patolsky, Y. Weizmann, I. Willner, *Angew. Chem. Int. Ed.* 43 (2004) 2113.
- [114] L.Y. Heng, A. Chou, W. Rahmat, M.N. Paddon-Row, J.J. Gooding, *Electroanalysis* 17 (2005) 38.
- [115] J. Huang, Y. Yang, H. Shi, Z. Song, Z. Zhao, J. Anzai, T. Osa, Q. Chen, *Mater. Sci. Eng. C* 26 (2006) 113.
- [116] G. Liu, Y. Lin, *Electrochem. Commun.* 8 (2006) 251.
- [117] H. Ju, H. Zhao, *Anal. Biochem.* 350 (2006) 138.
- [118] Y. Wang, P.P. Joshi, K.L. Hobbs, M.B. Johnson, D.W. Schmidtke, *Langmuir* 22 (2006) 9776.
- [119] X. Chu, D. Duan, G. Shen, R. Yu, *Talanta* 71 (2007) 2040.
- [120] M. Gao, L. Dai, G.G. Wallace, *Synth. Met.* 137 (2003) 1393.
- [121] S.G. Wang, Q. Zhang, R. Wang, S.F. Yoon, *Biochem. Biophys. Res. Commun.* 311 (2003) 572.
- [122] Y. Liu, M. Wang, F. Zhao, Z. Xu, S. Dong, *Biosens. Bioelectron.* 21 (2005) 984.
- [123] C. Cai, J. Chen, *Anal. Biochem.* 332 (2004) 75.
- [124] S. Hrapovic, D. Wang, F. Bensebaa, B. Simard, *Electroanalysis* 16 (2004) 132.
- [125] Y. Liu, L. Liu, S. Dong, *Electroanalysis* 19 (2007) 55.
- [126] N. Jia, L. Liu, Q. Zhou, L. Wang, M. Yan, Z. Jiang, *Electrochim. Acta* 51 (2005) 611.
- [127] Y.C. Tsai, S. Ci, S.W. Liao, *Biosens. Bioelectron.* 21 (2006) 495.
- [128] J. Wang, M. Musameh, *Anal. Chim. Acta* 539 (2009) (2005).
- [129] S. Qu, J. Wang, J. Kong, P. Yang, G. Chen, *Talanta* 71 (2007) 1096.
- [130] J.-S. Ye, Y. Wen, W.D. Zhang, H.F. Cui, G.Q. Xu, F.-S. Sheu, *Electroanalysis* 17 (2005) 89.
- [131] M. Zhang, A. Smith, W. Gorski, *Anal. Chem.* 76 (2004) 5045.
- [132] M. Yang, Y. Tang, H. Yang, G. Shen, R. Yu, *Biomaterials* 27 (2006) 246.
- [133] G. Li, J.M. Liao, G.Q. Hu, N.Z. Ma, P.J. Wu, *Biosens. Bioelectron.* 20 (2005) 2140.
- [134] S. Quiaocui, P. Tuzhi, Z. Yunu, C.F. Yang, *Electroanalysis* 17 (2005) 857.
- [135] X. Tau, M. Li, P. Cai, L. Luo, X. Zon, *Biochem. Anal.* 337 (2005) 111.
- [136] Z.-H. Gan, Q. Zhao, Z.-N. Gu, Q.-K. Zhuang, *Anal. Chim. Acta* 516 (2004) 29.
- [137] X. Cui, C.M. Li, J. Zang, Q. Zhou, Y. Gan, H. Bao, J. Guo, V.S. Lee, S.M. Mookhala, *J. Phys. Chem. C* 11 (2007) 2025.
- [138] G.-G. Zhao, L. Zhang, X.-W. Wei, *Anal. Biochem.* 329 (2004) 169.
- [139] G.C. Zhao, L. Zhang, X.-W. Wei, Z.-S. Yang, *Electrochem. Commun.* 5 (2003) 825.
- [140] G.C. Zhao, Z.-Z. Yin, L. Zhang, X.-W. Wei, *Electrochem. Commun.* 7 (2005) 256.
- [141] K. Yamamoto, G. Shi, T. Zhou, F. Xu, J. Xu, T. Kato, J.-Y. Jin, L. Jiu, *Analyst* 128 (2003) 249.
- [142] X. Yu, D. Chattopadhyay, I. Galeska, F. Papadimitrakopoulos, J.F. Rusling, *Electrochem. Commun.* 5 (2003) 408.
- [143] L. Qian, X. Yang, *Talanta* 68 (2006) 721.
- [144] L. Zhao, H. Liu, N. Hu, *J. Colloid Interface Sci.* 296 (2006) 204.
- [145] S. Chen, R. Yuan, Y. Chai, L. Zhang, N. Wang, X. Li, *Biosens. Bioelectron.* 22 (2007) 1268.

- [146] Y.-D. Zhao, Y.-H. Bi, W.-D. Zhang, Q.-M. Luo, *Talanta* 65 (2005) 489, 107 137.
- [147] Y. Li, X. Lin, C. Jiang, *Electroanalysis* 18 (2006) 2085.
- [148] L. Chen, G. Lu, *Sens. Actuators B* 121 (2007) 423.
- [149] V.S. Tripathi, V.B. Kandimalla, H. Ju, *Biosens. Bioelectron.* 21 (2006) 1529.
- [150] X. Luo, A.J. Killard, A. Morrin, M.R. Smyth, *Anal. Chim. Acta* 575 (2006) 39.
- [151] Y. Liu, M. Wang, F. Zhao, Z. Guo, H. Chen, S. Dong, *J. Electroanal. Chem.* 581 (2005) 1.
- [152] M. Wang, F. Zhao, Y. Liu, S. Dong, *Biosens. Bioelectron.* 21 (2005) 159.
- [153] Z. Xu, N. Gao, H. Chen, S. Dong, *Langmuir* 21 (2005) 10808.
- [154] S. Liu, Ch. Cai, *J. Electroanal. Chem.* 602 (2007) 103.
- [155] D. Du, X. Huang, J. Cai, A. Zhang, J. Ding, S. Chen, *Anal. Bioanal. Chem.* 387 (2007) 1059.
- [156] Z. Song, J.-D. Huuag, B.-Y. Wu, H.-B. Shi, J.-I. Anzai, Q. Chen, *Sens. Actuators B* 115 (2006) 626.
- [157] G. Liu, Y. Lin, *Anal. Chem.* 78 (2006) 835.
- [158] R.P. Deo, J. Wang, I. Block, A. Mulchandani, K.A. Joshi, M. Trojanowicz, F. Scholz, W. Chen, Y. Lin, *Anal. Chim. Acta* 530 (2005) 185.
- [159] R.K.A. Joshi, J. Tang, R. Haddon, J. Wang, W. Chen, A. Mulchandani, *Electroanalysis* 17 (2005) 54.
- [160] Y. Lin, F. Lu, J. Wang, *Electroanalysis* 16 (2004) 145.
- [161] G.A. Rivas, M.L. Pedano, *Electrochemical DNA biosensors*, in: Craig A. Grimes, Elizabeth C. Dickey, Michael V. Pishko (Eds.), *Encyclopedia of Sensors*, vol. 3, American Scientific Publishers, 2006, ISBN 1-58883-059-4, pp. 45–91.
- [162] M.L. Pedano, G.A. Rivas, *Electrochem. Commun.* 6 (2004) 10.
- [163] Y. Ye, H. Ju, *Biosens. Bioelectron.* 21 (2005) 735.
- [164] A. Ferancová, R. Ovádeková, M. Vanícková, A. Satka, R. Viglasky, J. Sima, J. Barek, J. Labuda, *Electroanalysis* 18 (2006) 163.
- [165] L.Y. Heng, A. Chou, J. Yu, Y. Chen, J.J. Gooding, *Electrochem. Commun.* 7 (2005) 1457.
- [166] J. Wang, A.-N. Kawde, M. Musameh, *Analyst* 128 (2003) 912.
- [167] J. Wang, G. Liu, M.R. Jan, *J. Am. Chem. Soc.* 126 (2004) 3010.
- [168] J. Wang, G. Liu, M.R. Jan, Q. Zhu, *Electrochem. Commun.* 5 (2003) 1000.
- [169] P. He, L. Dai, *Chem. Commun.* 6 (2004) 348.
- [170] H. Cai, Y. Xu, P.-G. He, Y.-Z. Fang, *Electroanalysis* 15 (2003) 1864.
- [171] H. Cai, X. Cao, Y. Jiang, P. He, Y. Fang, *Anal. Bioanal. Chem.* 375 (2003) 287.
- [172] N. Zhu, Z. Chang, P. Hem, Y. Fang, *Anal. Chim. Acta* 545 (2005) 21.
- [173] G. Cheng, J. Zhao, Y. Tu, P. He, Y. Fang, *Anal. Chim. Acta* 533 (2005) 11.
- [174] Y. Yang, Z. Wang, M. Yang, J. Li, F. Zheng, G. Shen, R. Yu, *Anal. Chim. Acta* 584 (2007) 268.
- [175] K. Kerman, Y. Morita, Y. Takamura, E. Tamiya, *Anal. Bioanal. Chem.* 381 (2005) 1114.
- [176] B. Munge, G. Liu, G. Collins, J. Wang, *Anal. Chem.* 77 (2005) 4662.
- [177] M. Guo, J. Chen, L. Nie, S. Yao, *Electrochim. Acta* 49 (2004) 2637.
- [178] P. He, M. Bayachou, *Langmuir* 21 (2005) 6086.
- [179] J. Li, Q. Liu, Y. Liu, S. Liu, S. Yao, *Anal. Biochem.* 346 (2005) 107.
- [180] J. Wang, *Analytical Electrochemistry*, second ed., Wiley-VCH, 2000.
- [181] M. O'Connor, S.N. Kim, A.J. Killard, R.J. Foster, M.R. Smyth, F. Papadimitrakopoulos, J.F. Rusling, *Analyst* 129 (2004) 1176.
- [182] D. Pantarotto, C.D. Partidos, R. Graff, J. Hoebeke, J.-P. Briand, M. Prato, A. Bianco, *J. Am. Chem. Soc.* 125 (2003) 6160.
- [183] J. Okuno, K. Maehashi, K. Kerman, Y. Takamura, K. Matsumoto, E. Tamiya, *Biosens. Bioelectron.* 22 (2007) 2377.
- [184] X. Yu, S.N. Kim, F. Papadimitrakopoulos, J.F. Rusling, *Mol. Biosyst.* 1 (2005) 70.
- [185] Y.H. Yun, A. Bange, W. Heineman, H.B. Halsall, V.N. Shanov, Z. Dong, S. Pixley, M. Behbehani, A. Jazieh, Y. Tu, D.K.Y. Wong, A. Bhattacharya, M.J. Schultz, *Sens. Actuators B* 123 (2007) 177.
- [186] S. Sánchez, M. Pumera, E. Fábregas, *Biosens. Bioelectron.* 23 (2007) 332.

Amperometric sensor for ethanol based on one-step electropolymerization of thionine–carbon nanofiber nanocomposite containing alcohol oxidase[☆]

Lina Wu^a, Mike McIntosh^b, Xueji Zhang^{b,c,*}, Huangxian Ju^{a,*}

^a Key Laboratory of Analytical Chemistry for Life Science (Education Ministry of China),

Department of Chemistry, Nanjing University, Nanjing 210093, China

^b Department of Chemistry, University of South Florida, 4202 East Fowler Avenue, CHE 305, Tampa, FL 33620-5250, USA

^c World Precision Instruments, Inc., Sarasota, FL 34240-9258, USA

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Abstract

Thionine had strong interaction with carbon nanofiber (CNF) and was used in the non-covalent functionalization of carbon nanofiber for the preparation of stable thionine–CNF nanocomposite with good dispersion. With a simple one-step electrochemical polymerization of thionine–CNF nanocomposite and alcohol oxidase (AOD), a stable poly(thionine)–CNF/AOD biocomposite film was formed on electrode surface. Based on the excellent catalytic activity of the biocomposite film toward reduction of dissolved oxygen, a sensitive ethanol biosensor was proposed. The ethanol biosensor could monitor ethanol ranging from 2.0 to 252 μM with a detection limit of 1.7 μM . It displayed a rapid response, an expanded linear response range as well as excellent reproducibility and stability. The combination of catalytic activity of CNF and the promising feature of the biocomposite with one-step non-manual technique favored the sensitive determination of ethanol with improved analytical capabilities.

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Keywords: Amperometric sensor; Electropolymerization; Carbon nanofiber; Thionine; Ethanol

1. Introduction

In recent years, the unique properties and potential technological applications of various nanomaterials, particularly carbon nanomaterials, have attracted increasing interest [1,2]. Carbon nanomaterials possess good electrical conductivity, unique structural and catalytic properties, high loading of biocatalysts, good mechanical and chemical stability, and excellent adsorption and penetrability [3–6]. Their functionalization has been a powerful strategy to improve the solubility or wettability, realize their potential in a wide range of application, and prepare novel materials with tailor-made structures and properties [7–9]. However, the covalent functionalization can often destroy the sp^2 structures thereby diminishing their pristine mechanical and electronic properties [10,11]. Thus, non-covalent functional strategy seems to be a more friendly and effective alternative for this purpose through effective π – π stacking [12], which has

been used to facilitate the solubility of carbon nanotubes (CNTs) [13–17]. Aromatic compounds such as methylene blue and toluidine blue have been used for the non-covalent functionalization of CNTs [12,13]. This work proposed a thionine–functionalized carbon nanofiber (CNF) for preparation of biosensors by electrochemical copolymerization of the functionalized CNF and oxidase.

CNF has cylindrical nanostructure with graphene layers arranged as stacked cones, cups, or plates [18]. The mechanical strength and electric properties of CNF are similar to CNTs [19]. This work showed that CNF also had a strong interaction with thionine molecule like CNTs. Non-covalent functionalization of CNF with the thionine molecule was very simple and useful to facilitate the solubility. Thionine–CNF nanocomposite could form a thin polymeric film on an electrode surface with an electropolymerization process, which could effectively incorporate enzyme into the film [20,21]. The electropolymerization process was easily controlled [22], and the film could provide a biocompatible surface to maintain the activity of entrapped enzyme [23]. With the incorporation of alcohol oxidase (AOD), an ethanol biosensor was thus prepared based on the electrocatalytic response of CNF toward the reduction of dissolved oxygen [24].

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* Corresponding authors. Tel.: +86 25 83593593; fax: +1 941 377 5428.

E-mail addresses: xueji@wpiinc.com (X. Zhang), hxju@nju.edu.cn (H. Ju).

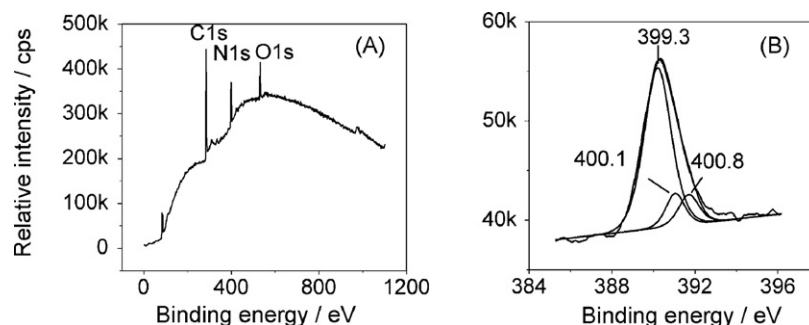


Fig. 1. (A) XPS spectrum and (B) N1s XPS spectrum of poly(thionine)-CNF film.

The quantitative detection of ethanol plays an important role in the quality control of alcoholic beverages during the fermentation process and clinical analyses of human breath and blood [25,26]. Ethanol amperometric biosensors are commonly based on either alcohol dehydrogenase (ADH) or alcohol oxidase (AOD) [27–30]. ADH based enzyme biosensor has the disadvantage that NAD^+ has to be added to the assay. The ethanol biosensor based on AOD are based on the monitoring of O_2 consumption or H_2O_2 formation, thus only dissolved oxygen is required as a co-substrate [31]. CNF has been used for the electrochemical detection of dissolved oxygen [32,33]. This work made use of the electrocatalytic reduction of CNF toward dissolved oxygen and the O_2 consumption at poly(thionine)-CNF/AOD film for ethanol detection. The proposed biosensor showed rapid response and excellent reproducibility and stability.

2. Experimental

2.1. Chemicals

CNF was a gift from WPI (Sarasota, USA). AOD from *Hansenula polymorpha* with an activity of 20–40 U/mg was purchased from Sigma. Thionine was from the Shanghai Biochemical Reagent (China). Other reagents were of analytical reagent grade. All solutions were prepared with twice-distilled water. The buffer for the assay was 0.2 M phosphate buffer saline (PBS), prepared by mixing stock standard solution of K_2HPO_4 and KH_2PO_4 .

2.2. Apparatus

Electrochemical measurements were performed on a CHI 660 electrochemical analyzer (Co. CHI, USA) with a conventional three-electrode system comprised of platinum wire as auxiliary electrode, saturated calomel electrode as reference and modified glassy carbon electrode (GCE) as working electrode. The cyclic voltammetric and amperometric experiments were performed in a thermostated electrochemical cell at 25 °C. Amperometric experiments were carried out in a stirring air-saturated 0.2 M pH 7.0 PBS by applying a potential step of -0.4 V.

UV-vis absorbance spectroscopy was performed using a UV-vis-3100-NIR recording spectrophotometer (Shimadzu,

Japan). X-ray photoelectron spectrum (XPS) was recorded on an ESCALAB MKII X-ray photoelectron spectrometer.

2.3. Construction of ethanol biosensor

The GCE was successively polished to a mirror finish using 0.3 and 0.05 μm alumina slurry (Beuhler) followed by rinsing thoroughly with twice-distilled water. After successive sonication in 1:1 nitric acid, acetone and twice-distilled water, the electrode was rinsed with twice-distilled water and allowed to dry at room temperature. 0.5 mg CNF was added into 1.0 mg/mL thionine solution and sonicated at room temperature for 1 h to obtain a well-dispersed thionine-CNF nanocomposite solution.

The electropolymerization was performed in the mixture of 4 mL thionine-CNF nanocomposite solution and 1 mL 0.2 M at +1.5 V for 300 s. The poly(thionine)-CNF film was then grown in the same solution by potential cycling between -0.5 and 0.0 V at 50 mV/s for 50 cycles. The poly(thionine)-CNF/AOD modified electrode was prepared through the same process in presence of 0.2 mg/mL AOD.

3. Results and discussion

3.1. XPS and UV-vis characterization of poly(thionine)-CNF film

XPS was used to investigate the surface composition of the polymer film prepared under optimal experimental conditions. The intense signals for C1s at 285 eV, N1s at 398 eV and O1s at 531 eV were observed on the XPS survey spectrum of the polymer film (Fig. 1A). The N1s spectrum could be well resolved into three peaks at 400.1, 399.3 and 400.8 eV (Fig. 1B), indicating that three chemical states of nitrogen atoms existed in the polymer film, which were in accordance with the poly(thionine) film [34]. The peak at 399.3 eV was corresponding to the amino nitrogen atoms, while the peak at 400.8 eV was ascribed to the heterocyclic nitrogen atom. The peak at 400.1 eV was ascribed to the bonding via an amino nitrogen atom in one thionine molecule to a carbon atom.

The atomic ratio of carbon to nitrogen could be used to estimate the surface composition of the polymer film. The results showed the polymer film contained 75.1 atomic% carbon and 14.6 atomic% nitrogen, thus the calculated weight percents of thionine was 84.2% and CNF was 15.8%. The weight ratio of

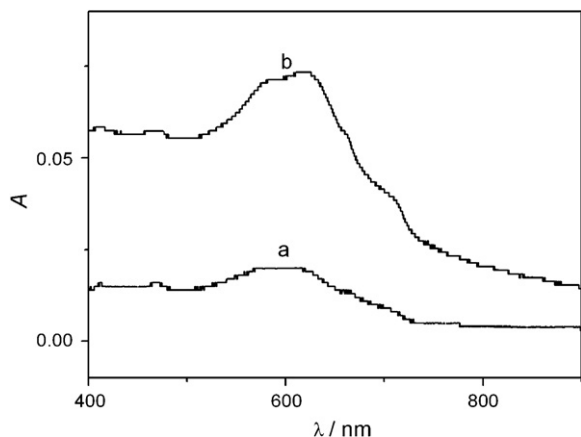


Fig. 2. UV-vis spectra of (a) poly(thionine) and (b) poly(thionine)-CNF composite.

thionine to CNF was higher than that in electropolymerization solution, which indicated that more thionine molecules were participated in the electropolymerization.

Fig. 2 shows the UV-vis spectra of poly(thionine) and poly(thionine)-CNF film. Poly(thionine) showed an absorption peak in the visible region around 600 nm, which showed a little red shift observed in poly(thionine)-CNF, indicating that CNF was incorporated into the film and interacted with thionine molecules.

3.2. Electropolymerization of thionine-CNF nanocomposite

The preparation of poly(thionine)-CNF film was performed using the method reported for the electropolymerization of thionine in neutral aqueous solution [34]. The growth of poly(thionine)-CNF film was dependent on the preanodization operation at a constant potential of +1.5 V carried out on a bare GCE in pH 7.0 PBS containing thionine-CNF nanocomposite. A large amount of positive charge could be accumulated to create the thionine cation radical during this process. After the preanodization, the poly(thionine)-CNF film was gradually formed with successive cyclic sweeps, as indicated in Fig. 3. During the process of electropolymerization, a pair of reversible redox peaks were observed at -0.268 and -0.246 V, which were attributed to the oxidation and reduction of thionine unit. Both the cathodic and anodic peak currents increased gradually and trended towards the stable values. The peak shape also became better, indicating the gradually increasing coverage of thionine unit. Finally, a stable cyclic voltammogram with almost symmetric shape was obtained, which was stable in pH 7.0 PBS upon cyclic sweep at 50 mV/s over the same potential range.

Fig. 4 shows the cyclic voltammograms of the poly(thionine)-CNF modified GCE at various scan rates. Both the anodic and cathodic peak currents were proportional to the scan rate in the range from 10 to 400 mV/s, indicating a surface-controlled electrode process. The surface coverage was calculated from the peak areas to be $(4.42 \pm 0.15) \times 10^{-9}$ mol/cm².

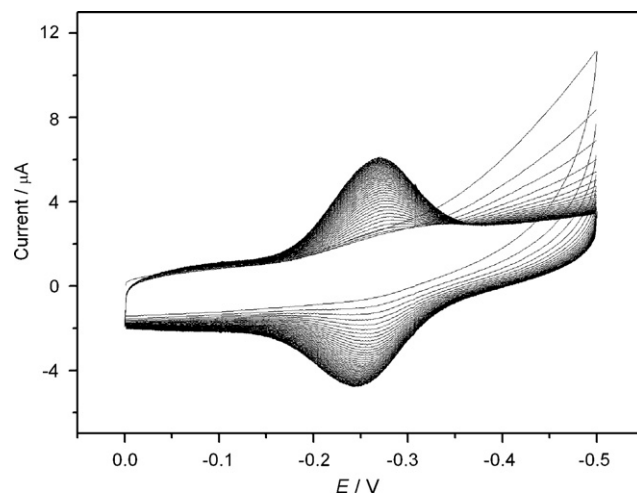


Fig. 3. Cyclic voltammogram for electropolymerization of CNF-thionine nanocomposite on pretreated GCE after preanodization at +1.5 V for 300 s in pH 7.0 PBS containing thionine-CNF nanocomposite.

3.3. Electrocatalysis of poly(thionine)-CNF toward reduction of dissolved oxygen

Fig. 5 displays the cyclic voltammograms of poly(thionine)-CNF modified GCE in nitrogen- and air-saturated 0.2 M pH 7.0 PBS. In the potential window from 0 to -0.5 V, the poly(thionine)-CNF modified GCE exhibited a pair of small redox peaks at -0.251 and -0.271 V at 50 mV/s in nitrogen-saturated PBS. In air-saturated PBS, the reduction peak current increased from 5.80 to 7.78 μ A and the oxidation peak current decreased, indicating a typical electrocatalytic reduction process toward dissolved oxygen. The poly(thionine) modified electrode did not show observable electrocatalytic activity toward the reduction of dissolved oxygen, so the electrocatalytic activity should be ascribed to the CNF whose electrocatalytic property had been reported in [24].

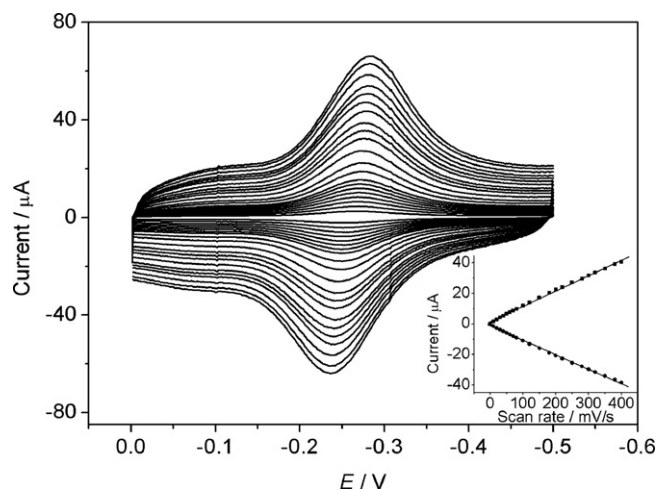


Fig. 4. Cyclic voltammograms of poly(thionine)-CNF modified GCE in 0.2 M pH 7.0 PBS at 10, 20, 30, 40, 50, 60, 70, 80, 100, 120, 150, 180, 200, 220, 250, 280, 300, 320, 350, 380 and 400 mV/s. Inset: plots of peak currents vs. v .

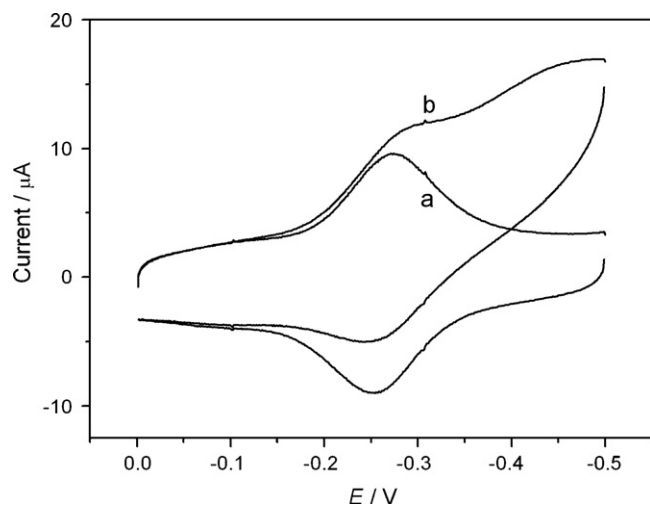


Fig. 5. Cyclic voltammograms of poly(thionine)–CNF modified GCE in 0.2 M pH 7.0 nitrogen: (a) and air-saturated (b) PBS.

3.4. Electropolymerization of thionine–CNF nanocomposite containing AOD

The isoelectric point of AOD is 6.1. In pH 7.0 PBS, the enzyme was negatively charged. Since the thionine–CNF nanocomposite was polymerized as a polycation of thionine, the AOD could be incorporated into the growing film via electrostatic affinity interaction. The cyclic voltammogram for electrochemical polymerization of thionine–CNF/AOD nanocomposite film in 0.2 M pH 7.0 PBS containing 0.2 mg/mL AOD after preanodization is shown in Fig. 6A. During the electropolymerization process, the peak currents of a pair of reversible redox peaks at -0.288 and -0.231 V increased. In comparison with Fig. 2, the peak-to-peak separation was larger, indicating that AOD was incorporated in the polymer film and blocked the electron transfer.

The concentration of AOD in electropolymerization solution was an important parameter for substrate detection. With the increasing concentration of enzyme in electropolymerization solution, the response to ethanol increased and then tended towards a constant value. At the enzyme concentration of 0.2 mg/mL, the current reached a maximum value, indicating a saturation of enzyme in the electropolymerized film.

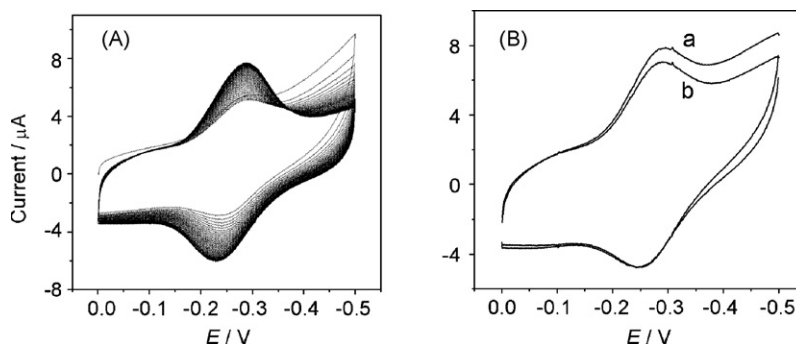


Fig. 6. Cyclic voltammograms for (A) coimmobilization of AOD and CNF-thionine nanocomposite on pretreated GCE after preanodization at +1.5 V for 300 s in pH 7.0 PBS containing thionine–CNF composite and 0.2 mg/mL AOD and (B) poly(thionine)–CNF/AOD modified GCE in pH 7.0 air-saturated PBS in absence (a) and presence (b) of 10 mM ethanol.

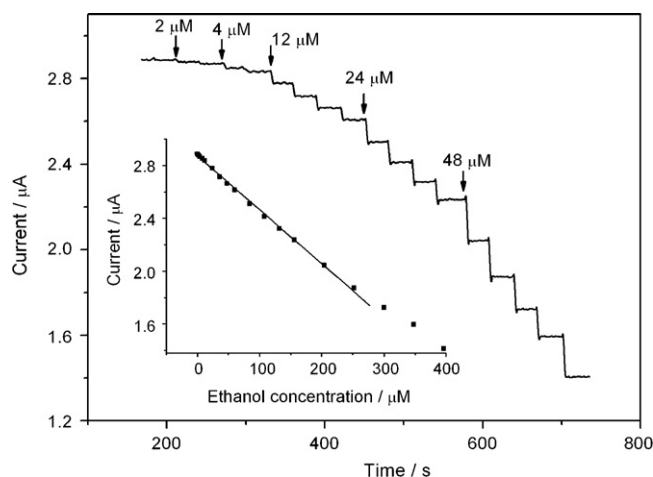


Fig. 7. Successive amperometric responses of the poly(thionine)–CNF/AOD modified GCE to ethanol in pH 7.0 PBS at -0.4 V. Inset: plot of amperometric response vs. concentration of ethanol.

When the concentration was larger than this value, excess AOD could not be incorporated in the polymer film and would block the electron exchange. Thus, the concentration of 0.2 mg/mL AOD was used for preparation of poly(thionine)–CNF/AOD film.

3.5. Amperometric biosensing of ethanol

AOD is an oligomeric flavoprotein with eight identical subunits arranged in a quasi-cubic orientation, each containing a non-covalently bound flavin adenine dinucleotide molecule (FAD) as a cofactor [26]. AOD can catalyze the oxidation of ethanol by O_2 into the corresponding aldehydes with the concomitant production of H_2O_2 [26]. During this reaction, the AOD cofactor (FAD) is first reduced to its hydrogenated form (FADH_2) and re-oxidized to its native form [35]. Ethanol biosensors are generally based on the detection of the oxidation signal of H_2O_2 or the reduction signal of O_2 . The poly(thionine)–CNF/AOD based biosensor retained the catalytic activity of the CNF for the efficient reduction of dissolved oxygen, allowing fast and sensitive ethanol quantification.

As shown in Fig. 6B, in the potential window from 0 to -0.5 V, the poly(thionine)–CNF/AOD modified GCE exhibited

a pair of redox peaks at -0.249 and -0.292 V at 50 mV/s in air-saturated PBS. Upon addition of ethanol to the air-saturated PBS, the reduction current response of poly(thionine)–CNF/AOD modified GCE decreased from 4.01 to 3.56 μ A due to the consumption of dissolved oxygen.

Fig. 7 shows the amperometric response of the poly(thionine)–CNF/AOD modified GCE at -0.4 V to the successive addition of ethanol in 0.2 M pH 7.0 PBS. Immediately after the addition of ethanol, the current decreased and reached a steady state within 5 s. The response displayed a linear range from 2.0 to 252 μ M with a correlation coefficient of 0.999 and a slope of 4.1 nA/ μ M. The detection limit is 1.7 μ M, which was much lower than that of 6.26 mM ($3.5 \times 10^{-2}\%$, v/v) based on AOD immobilized within electrochemically deposited resydrol film [36]. The sensitivity was 6.8 times higher than that of 0.6 nA/ μ M at a trienzyme graphite–Teflon–glucose oxidase–AOD–horseradish peroxidase–ferrocene based biosensor [37].

The coefficients of variation for intra-assay with this method were 5.9% and 5.0% at the ethanol concentrations of 8.0 and 80 μ M, respectively. In addition, the relative standard deviation of current signals for the measurement of 100 μ M ethanol at five independently prepared biosensors was 8.2% , which proved good reproducibility of the biosensor preparation.

The stability of the poly(thionine)–CNF/AOD modified electrode was investigated when stored in 0.2 M pH 7.0 PBS at 4°C . After 4 weeks the response current was still retained at 96% of the initial response, implying that the three-dimensional structure of the poly(thionine)–CNF structure was very efficient for retaining the bioactivity of AOD.

4. Conclusions

A nanocomposite of thionine–CNF with good dispersion in water can be prepared through π – π electronic and hydrophobic interaction of thionine and CNF. With a one-step electrochemical polymerization two nanocomposite films of poly(thionine)–CNF and poly(thionine)–CNF/AOD have been successfully immobilized on an electrode surface. The presence of CNF leads to excellent catalytic activity of the nanocomposite films to the reduction of dissolved oxygen, producing a sensitive ethanol biosensor. The proposed ethanol biosensor possesses excellent characteristics and performance such as low detection limit, fast response and good stability, manifesting that the as-prepared thionine–CNF nanocomposite and this electrochemical polymerization method are suitable for enzyme immobilization and biosensor construction.



Dear Professor Wang,

It is a great honor and a tremendous pleasure for me to congratulate you on your 60th birthday!

When I was a postdoc in your lab at NMSU, you helped shepherd me through, and I will never forget the encouragement and guidance you gave me. Your life has been an inspiration for many scholars, but it touched mine in very special ways. You inspired me at a turning point in my life. There are so many ways that I learned from your example, but perhaps the most important lesson was your belief in the importance of what you were doing. You always tell us “no pain, no gain”. I only hope I can repay some of my debt to you by passing on some of the lessons I learned to my students and colleagues who have not had the good fortune of leaning from you and knowing you. Most are admired because they move others to follow their example . . . only a very few are special enough to encourage others to find their own unique path. Just by being who you are, you have sparked me to be who I am. And for that, if nothing else, I call you my inspiration.

Happy Birthday, Professor Joseph Wang! May your bright light shine on! May it be for you a wonderful, flourishing, exciting decade! (By Xueji Zhang).



Dear Joe,

Congrats and best wishes on your 60th birthday, another milestone another success. As your collaborator, I wish your health be great and your birthdays. In oriental philosophy, 60 years means the completion of one full life cycle and the beginning of a new cycle. Hence, a 60th birthday is a very significant event and a time for great celebrations. On your 60th anniversary, I wish to extend my special congratulations to you, a leader in the development of many chemical and biological sensors, striving as always for further advances in our field. Your important works on behalf of Electroanalysis and Sensors have benefited many people a great deal, both within and outside academia. You will probably never know how many people like me deeply appreciate you and celebrate your creativity and wisdom.

I am sure that anyone has lived those 60 years in a manner as remarkable and distinguished as you have done is indeed a cause for celebration. Happy Birthday to you Joe! (by Huangxian Ju).

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ural Science Foundation of China (20535010) and NIH grant (5R44GM62077-03).

References

- [1] M. Yemini, M. Reches, E. Gazit, J. Rishpon, *Anal. Chem.* 77 (2005) 5155.
- [2] S.S. Wong, E. Joselevich, A.T. Wooley, C.L. Cheung, C.M. Leiber, *Nature* 394 (1998) 52.
- [3] D. Lee, J. Lee, J. Kim, J. Kim, H.B. Na, B. Kim, C.-H. Shin, J.H. Kwak, A. Dohnalkova, J.W. Grate, T. Hyeon, H.-S. Kim, *Adv. Mater.* 17 (2005) 2828.
- [4] O. Niwa, *Bull. Chem. Soc. Jpn.* 78 (2005) 555.
- [5] S. Boussaad, N.J. Tao, T. Hopson, L.A. Nagahara, *Chem. Commun.* 13 (2003) 1502.
- [6] P.G. He, L.M. Dai, *Chem. Commun.* 3 (2004) 348.
- [7] K. Wang, W.W. Li, C. Gao, *J. Appl. Polym. Sci.* 105 (2007) 629.
- [8] K.P. De Jong, J.W. Geus, *Catal. Rev. Sci. Eng.* 42 (2000) 481.
- [9] D. Tasis, N. Tagmatarchis, A. Bianco, M. Prato, *Chem. Rev.* 106 (2006) 1105.
- [10] Q.W. Li, J. Zhang, H. Yan, M.S. He, Z.F. Liu, *Carbon* 42 (2004) 287.
- [11] R. Niyogi, M.A. Hamon, R.C. Haddon, *Acc. Chem. Res.* 35 (2002) 1105.
- [12] Y.M. Yan, M.N. Zhang, K.P. Gong, L. Su, Z.X. Guo, L.Q. Mao, *Chem. Mater.* 17 (2005) 3457.
- [13] Y. Liu, J.P. Lei, H.X. Ju, *Talanta*, in press.
- [14] A. Star, T.-R. Han, J.-C.P. Gabriel, K. Bradley, G. Gruñner, *Nano Lett.* 3 (2003) 1421.
- [15] Y. Sun, S.R. Wilson, D.I. Schuster, *J. Am. Chem. Soc.* 123 (2001) 5348.
- [16] M.F. Islam, E. Rojas, D.M. Bergey, A.T. Johnson, A.G. Yodh, *Nano Lett.* 3 (2003) 269.
- [17] A. Star, J.F. Stoddart, D. Steuerman, M. Diehl, A. Boukai, E. Wong, X. Yang, S.-W. Chung, H. Choi, J.R. Heath, *Angew. Chem. Int. Ed.* 40 (2001) 1721.
- [18] A. Arvinte, F. Valentini, A. Radoi, F. Arduini, E. Tamburri, L. Rotariu, G. Palleschi, C. Balaa, *Electroanalysis* 19 (2007) 1455.
- [19] M. Endo, Y.A. Kim, T. Fukai, T. Hayashi, K. Oshida, M. Terrones, T. Yanagisawa, S. Higaki, M.S. Dresselhaus, *Appl. Phys. Lett.* 80 (2002) 1267.
- [20] K.A. Marx, T. Zhou, *J. Electroanal. Chem.* 521 (2002) 53.
- [21] Y. Xiao, H.X. Ju, H.Y. Chen, *Anal. Chim. Acta* 391 (1999) 299.
- [22] C. Hao, L. Ding, X.J. Zhang, H.X. Ju, *Anal. Chem.* 79 (2007) 4442.
- [23] D.D. Long, K.A. Marx, T. Zhou, *J. Electroanal. Chem.* 501 (2001) 107.
- [24] L.N. Wu, X.J. Zhang, H.X. Ju, *Biosens. Bioelectron.* 23 (2007) 479.
- [25] O. Smutok, B. Ngounou, H. Pavlishko, G. Gayda, M. Gonchar, W. Schuhmann, *Sens. Actuat. B Chem.* 113 (2006) 590.
- [26] H.B. Yildiz, L. Toppare, *Biosens. Bioelectron.* 21 (2006) 2306.
- [27] E. Akyilmaz, E. Dinckay, *Talanta* 53 (2000) 505.
- [28] E. Akyilmaz, E. Dinckaya, *Talanta* 61 (2003) 113.
- [29] L.N. Wu, X.J. Zhang, H.X. Ju, *Anal. Chem.* 79 (2007) 453–458.
- [30] Z.H. Dai, G.F. Lu, J.C. Bao, X.H. Huang, H.X. Ju, *Electroanalysis* 19 (2007) 1591.
- [31] A.M. Azevedo, D.M.F. Prazeres, J.M.S. Cabral, L.P. Fonseca, *Biosens. Bioelectron.* 21 (2005) 235.
- [32] S. Maldonado, K.J. Stevenson, *J. Phys. Chem. B* 108 (2004) 11375.
- [33] S. Maldonado, K.J. Stevenson, *J. Phys. Chem. B* 109 (2005) 4707.
- [34] R. Yang, C.M. Ruan, W.L. Dai, J.Q. Deng, J.L. Kong, *Electrochim. Acta* 44 (1999) 1585.
- [35] A.M. Azevedo, J.M.S. Cabral, T.D. Gibson, L.P. Fonseca, *J. Mol. Catal. B: Enzym.* 28 (2004) 45.
- [36] L.V. Shkotova, A.P. Soldatkin, M.V. Gonchar, W. Schuhmann, S.V. Dzyadevych, *Mater. Sci. Eng. C* 26 (2006) 411.
- [37] A. Guzman-Vázquez de Prada, N. Peña, C. Parrado, A.J. Reviejo, J.M. Pingarrón, *Talanta* 62 (2004) 896.