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Enzyme/semiconductor nanoclusters combined systems for novel amperometric biosensors

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Abstract

In this work quantum-sized CdS nanocrystals were synthesized using a quaternary water-in-oil microemulsion and immobilized onto gold working electrode by self-assembled monolayers techniques. Formaldehyde dehydrogenase was covalently immobilized onto a protecting membrane, which was stratified on part of the semiconductor nanoparticles modified electrode. The covalent enzyme immobilization has been required to improve the stability of the catalytic oxidation of formaldehyde, which occurs after light stimulation of the semiconductor through the electron/hole recombination. A study about the best electrochemical oxidation potentials under different flow conditions was performed. Preliminary sensor stability and interferences tests were also carried out, for a sensitive and selective detection of formaldehyde. A detection limit of 41 ppb of formaldehyde was calculated and an operational stability of 6 h was achieved under flow conditions by means of this novel amperometric biosensor based on FDH-semiconductor hybrid systems, not requiring NAD^+/NADH as charge transfer in the enzymatic reaction.

Keywords: Formaldehyde dehydrogenase; Enzyme-semiconductors hybrid; CdS nanoparticles; Amperometric biosensor

1. Introduction

Nanostructured materials have proven as one of the most powerful tool in new trend of technology and research, due to their absolutely peculiar properties at nanometer size scale. Many studies have shown that optical, mechanical, photocatalytic and transport properties drastically changes, depending on quantum size effect, as the mean diameter of the particles is in the exciton size regime (i.e. <10 nm) [1–9]. Nanosized semiconductors find large applications in luminescence, non-linear optic, catalysis, optoelectronics and photochemistry [10–13].

New combined structures made of semiconductor NCs and enzymes have been already designed. In such systems electron and hole generated respectively in the conduction and valence bands can be used for the substrate reduction (oxidation) through electron acceptor (donor). The electronic transfer mechanism of hole and electron towards the interface enzyme/NCs depends strongly upon the redox potential of semiconductor, the enzymatic reaction and the presence of a substance, which can be reversibly oxidized and reduced.

Nanoparticles architectures on electrode supports pushed towards the development of electronic nano-devices such as in metal/semiconductor arrays assembled on electrodes, multi enzymatic arrays or biochips [14–17]. Extensive research has been also devoted to the surface activation, by organic monolayers, chemicophysical modification or

Abbreviations: CTAB, hexadecil trimethyl ammonium bromide; FDH, formaldehyde dehydrogenase; GA, glutaraldehyde; LOD, limit of detection; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); NCs, nanoclusters; PB, phosphate buffer 0.1 M (pH = 8.0); POH, pentanol; RE, reference electrode (Ag/AgCl); SAMs, self-assembled monolayers; WE, working electrode; w/o, water-in-oil

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biomaterials immobilization procedure [18–21]. The concept of self-assembled monolayers has been extended to insert metal and/or nanosized semiconductors onto several surfaces [22–30].

Formaldehyde is a commercially widespread chemical, largely produced for domestic, industrial and clinical uses, which is naturally present in fruits, vegetables, flesh, fishes, biological fluids. Formaldehyde detection became an environmental and clinical issue, because of its toxicological effect for consumers and proved mutagenic effects on several living organisms [31,32]. Most of the main established analytical methods for formaldehyde detection, including visible absorption [33], liquid chromatography (HPLC, IC) [34–36], gas chromatography [37–38] and fluorimetry [39] reached high sensitivity but basically they require toxic reagent and skilled personnel, especially for the sample treatment, resulting in impractical real-time measurements. Recent efforts have turned towards the development of rapid and specific electrochemical biosensors based on NADH-dependent dehydrogenase, using different mediators for NADH electrooxidation either in batch reactors or under flow conditions [40–47].

Unfortunately, the NAD^+/NADH couple generally exhibits hard electrochemical oxidation of NADH. The most redox NAD^+/NADH dependent enzymes reveal fundamental difficulties, due to their poor stability, high costs and electrical contacting with electrode. Hence, semiconductor nanocrystals represent a possible route to enhance the electron/hole transfer efficiency, overcoming the problems due to the NAD^+/NADH dependence.

We attempted to use CdS nanosized semiconductors as new charge transfer agents in cofactor-dependent enzyme reactions, in order to construct novel amperometric biosensors for online formaldehyde monitoring. Nanosized semiconductors coupled to formaldehyde dehydrogenase (FDH) enzyme resulted in a hybrid system able to achieve the electron transport towards or from the electrode surface after suitable photo-activation. Curri et al. [48] already presented an enzyme-semiconductor hybrid, where the FDH enzymatic behavior was compared in water and micellar solution, investigating the enzymatic activity in the presence of semiconductor nanoparticles. Kinetic measurements carried out in microemulsive systems confirmed the enzymatic activity is fully retained in different ambient, especially in quaternary microemulsion, where a sufficient variety of (II–VI) semiconductors can be easily synthesized, without resulting in a denaturation of the enzyme properties. The successful enzymatic oxidation of formaldehyde under illumination was also demonstrated in FDH-semiconductor hybrid, as well as the reversibility of the reaction [48]. On the other hand, the low stability of the electrochemical response after each sequence of illumination still remains a limitation, owing to an observable photo-corrosion effect and to the possible inhibition of the enzymatic redox site.

Our purpose was to develop the presented hybrid system for more stable formaldehyde detection, without using the NAD^+/NADH couple for the charge transfer. Covalent immobilization of FDH on protecting functionalized membranes combined with semiconductor-based electrode was required to avoid the enzyme denaturation in solution, especially when using flow conditions. The influence of other redox substances was also investigated. The sensitivity of this novel system cannot be considered as practically comparable with respect to previous NAD-dependent biosensors for formaldehyde detection.

2. Experimental

2.1. Chemicals and reagents

Formaldehyde dehydrogenase (FDH) from *Pseudomonas putida* (EC 1.2.1.46, with specific activity between 4 and 6 units per mg), formaldehyde (stock solution: 4% w/v), glucose, glycine, GA (25% aqueous solution, solid), paracetamol, methanol, L(+)-ascorbic acid, sodium sulphite salt and cysteine were purchased from Sigma. Pre-activated Nylon ID membranes (Immunodyne™) were purchased from Pall Italia (Milan, Italy) and used according to the manufacturer's instructions. Buffer solution was prepared immediately before using. Cetyl-trimethyl ammonium bromide (CTAB) surfactant, pentanol, hexane, hexanedithiol, cadmium nitrate, sodium sulphide, potassium and sodium phosphate salts were from Merck, all other chemical were used without further purification and were of analytical grade. All reagents and electrolyte solution were prepared using twice distilled water.

2.2. Nanocrystals synthesis and immobilization on gold

Enormous improvements has been made in preparing and characterizing nanosized semiconductors of II–VI, III–V and IV type, metal doped or undoped, by means of host–guest inclusion chemistry or colloidal synthesis [49,50]. New synthetic methods are based on thermal decomposition of precursors in coordinating solvents and capping agents at relatively high temperature [51–55]. Nanostructured CdS preparation in quaternary water-in-oil microemulsive systems has been showed as one of the most simple and successful synthetic route, as extensively reported in several papers [56–61], to regulate at will the semiconductor particle size and properties, due to the simplicity of the synthesis and the well defined properties of the reverse micelles. In this paper CdS nanoparticles were prepared using a quaternary w/o microemulsion, formed by CTAB, as cationic surfactant, pentanol (the co-surfactant), *n*-hexane and water, mixing equivalent amounts of precursor salts in micellar solution, i.e. cadmium nitrate and sodium sulphide. The nanocrystals size were modulated and controlled by varying respectively water to surfactant ($W_0 = H_2O/CTAB$) and pentanol to surfactant ($P_0 = POH/CTAB$) ratios. In fixed experimental conditions (CTAB at 0.1 M, pentanol content in the range between 8

and 14, water-to-surfactant ratio in the range 10–80) it was demonstrated that the system consists of spherical reverse micelles, where it is possible to modulate the water droplets dimension and surface dynamics either varying W_0 or P_0 [58].

The immobilization of CdS nanocrystals on the working electrode was performed according to the self assembly (SAMs) techniques, immerging the gold substrate in a solution of 1,6-hexanedithiol and then exposing the resulting support to the CdS nanocluster microemulsive solution. The complete description of the used process is reported elsewhere [59,62,63]. The effectiveness of the reverse micelles synthesis for obtaining stable NCs layers, keeping their original size and individuality after the immobilization on gold support was already demonstrated [57,59]. SAMs deposition and CdS immobilization were also widely investigated by surface characterization techniques [59].

Experimentally, the probability of recombination between electron and hole in photo-activated semiconductors is proportional to the number of the nanostructured layers onto the electrode: a strong increase in the number of CdS layers generate a drop of photoanodic peaks, therefore another key point for the construction of highly ordered semiconductor nanocrystals is the careful control of the SAMs deposition on the gold substrate.

2.3. Enzyme immobilisation

FDH was immobilized covalently onto a pre-activated Nylon ID membrane (Immunodyne^{TM}), dropping a solution of 5 μL of the enzyme solution (65 mg mL^{-1} in PB 0.1 M) onto a little portion corresponding to a maximum area of 1 cm^{-2}. The membrane was dried in air and then immersed into a blocking solution of glycine 0.1 M in PB. After a washing procedure the membrane was stored at 4 °C ready to be used on the working nanostructured CdS/gold electrode. The working area must be larger than the selective enzymatic membrane, in order to enable the incoming light to activate the charge transfer.

2.4. Measuring set-up

For photo-electrochemical studies on the combined system, chrono-amperometric techniques were mainly used, applying a controlled tuneable potential simultaneously during the illumination of the semiconductor layers under flow injection conditions. The amperometric homemade flow cell consists of an 'inox' steel support onto which were placed the WE (CdS deposited on gold) and two Plexiglas plates on the upper side, where a circular groove was created for flow control (Fig. 1). Both a platinum counter electrode and a Ag/AgCl/KCl reference electrodes were electrically connected to the plates. As shown in Fig. 1, additional grooves were carved to assure that an optical fibre tungsten lamp (250 W) was directly lodged into the cell, resulting in a well defined light path through the grooves toward the semiconductor-based WE. The covalently bonded enzyme membrane was fixed by an O-ring between the Plexiglas plate and the WE, according to previous specifications: the working area must be larger than the membrane. The flow cell was connected to a peristaltic pump and to an injector equipped with a 5 μL loop. Photo-electrochemical measurements were carried out by a PC-controlled Autolab PGSTAT10 potentiostat. Known amounts of formaldehyde in buffer solution were injected into the cell at room temperature (20 ± 2 °C).

3. Results and discussion

3.1. Adjustment of the experimental parameters

Fig. 2 shows the current dependence on the flow rate using the three electrode flow cell system depicted in Fig. 1 at the applied potential of 78 mV versus Ag/AgCl RE, keeping as a constant the injected formaldehyde concentration. The best flow condition in terms of the signal sensitivity was reached at 0.3 mL min^{-1}; therefore all the following experiments were performed using this flow rate value. Fig. 3 suggests that the highest signal was reached at the working potential of 80 mV

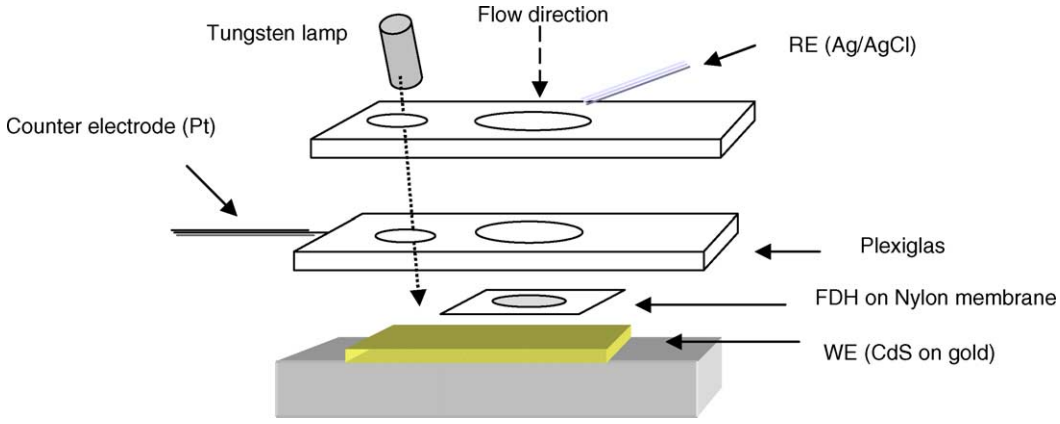

Fig. 1. Schematic picture of the three-electrode flow cell for photo-electrochemical measurements. The light path from the lamp towards the working electrode is also represented.

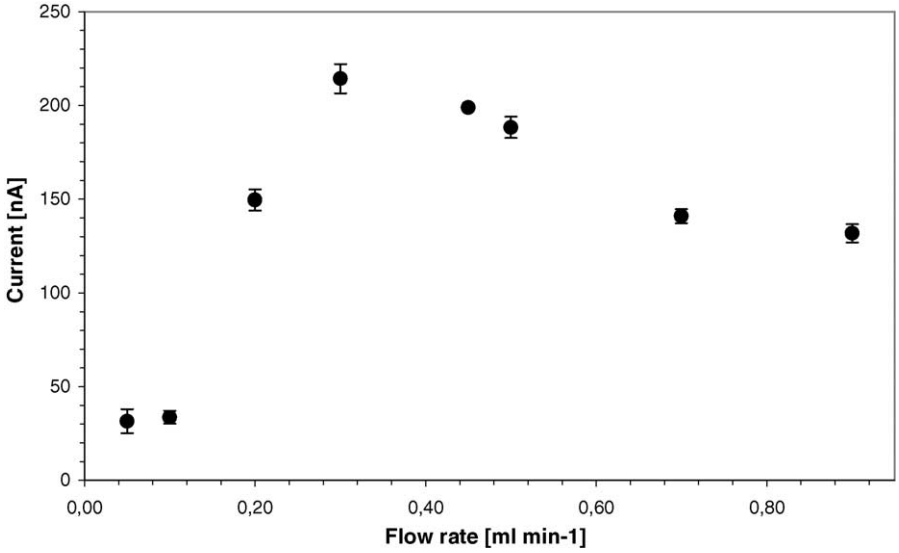

Fig. 2. Current response as a function of the flow rate at an applied voltage of 78 mV vs. Ag/AgCl RE using FDH/semiconductor NCs/gold sensor in the homemade flow detection cell. Injected formaldehyde solution: 5 μg mL^{-1}.

versus Ag/AgCl RE, injecting a concentration of 5 μg mL^{-1} of formaldehyde in PB solution.

Calibration curves were obtained after successive additions of formaldehyde under continuous illumination of the WE at 78 mV versus Ag/AgCl RE. As expected in common enzymatic reactions, the current response showed a linear relation only at low formaldehyde concentrations, i.e. up to 1 μg mL^{-1}. Linear dynamic range was achieved in the range 0.05–1 μg mL^{-1} (regression curve: $y = 0.0921x + 3.0972$, $r^2 = 0.994$ at the selected confidence level $\alpha = 0.05$, number of replicates $n = 4$). As indicated in the calibration graph of Fig. 4, a detection limit of 41 ng mL^{-1} was calculated at a signal-to-noise ratio of 3 according to the Zund–Meier graphical method for LOD determination [64].

3.2. Enzymatic photo-activity with CdS: operational stability of the sensor

In Fig. 5 the current signal as a function of time is reported as an example, using an enzyme membrane on the CdS-modified electrode under illumination. The optimal constant flow rate and potentiostatic conditions were studied in the previous section. The resulting signal is generated from the photo-activation of the CdS semiconductor to shuttle the electron in the enzyme-catalyzed oxidation of formaldehyde; all the experiments were made in the absence of NAD^+ cofactor, which is commonly used as charge mediator. The initial current increases between 2.9 and 3.3 μA is simply due to the injection of the substrate (formaldehyde at a fixed concentration). Under substrate injection immediately after the

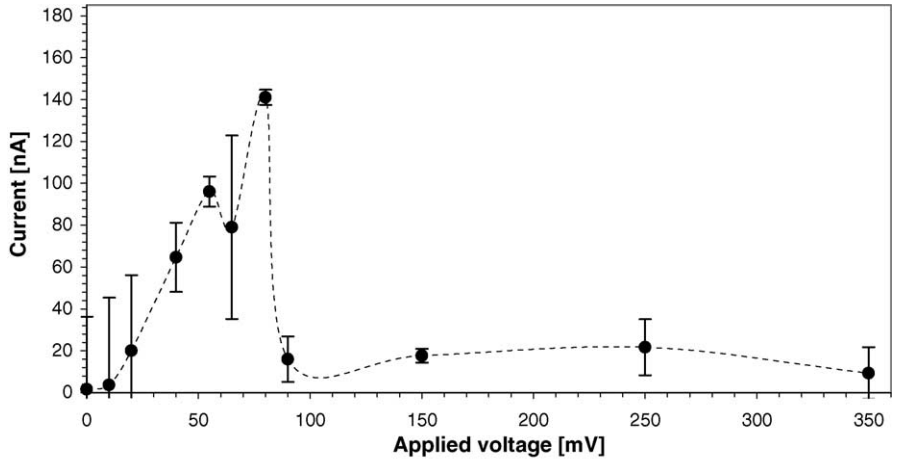

Fig. 3. Hydrodynamic voltammogram obtained varying the applied voltage vs. Ag/AgCl RE according to the experimental conditions reported in the text, using FDH/semiconductor NCs/gold sensor. Injected formaldehyde solution: 5 μg mL^{-1}.

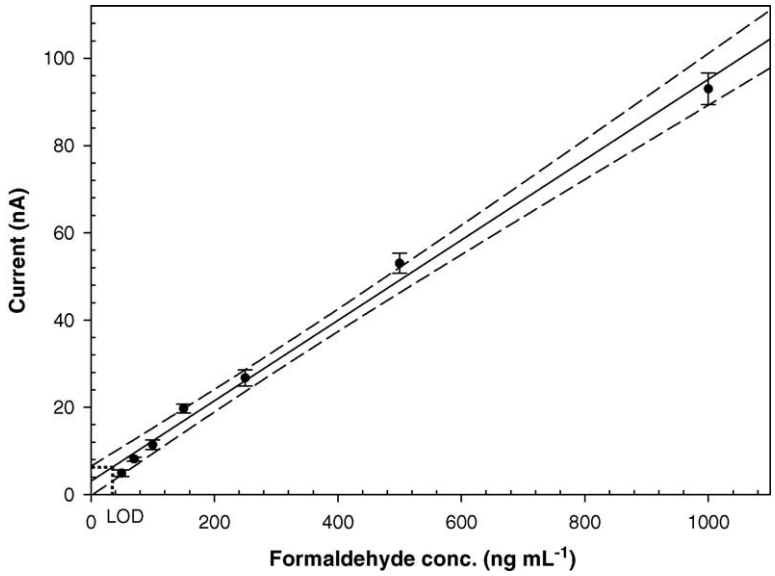

Fig. 4. Calibration curve of formaldehyde using FDH/semiconductor NCs/gold sensor in homemade flow detection cell. See the text for the experimental conditions. It is also reported the LOD evaluated with the Zund–Meier method.

illumination (on), the expected current variation occurred, reaching a readily constant value, and then returning to the initial background value in darkness conditions (black curve in Fig. 5). Photo-anodic currents showed an exponential behavior of a typical charging/discharging process. This trend is slower than a simple capacitive electrode process, because of diffusion effect of the semiconductor charge carriers. In order to ascribe the observed photo-anodic peaks to the specific enzyme catalyzed process, blank experiments were performed replacing the enzyme-based membrane with a nude activated membrane: no signal was detected in the absence of FDH (grey curve in Fig. 5). All such results indicate that nano-sized CdS semiconductor should function as mediator in the dehydrogenase-based oxidation of formaldehyde.

Further experiments with respect to the stability of the FDH/CdS/gold hybrid system were carried out. Operational stability were measured under continuous injection of a fixed amount of formaldehyde (0.5 μg mL^{-1}) determining the resulting current by the sequential illumination during a period of more than 12 h at a frequency of 12 illumination cycles per hour. Signal stability better than 90% was achieved after stabilization (data not shown): a relatively stable peak height was kept at least for the first 6 h, with a signal enhancement comparing to the previous work based on semiconductor FDH/CdS/gold systems [48].

3.3. Selectivity of the sensor

The selectivity of many amperometric biosensors is often restricted by the anode oxidation of interferents whose reactions can cause electrode fouling and poisoning. In complex medium or bioorganic matrices where many reactions take

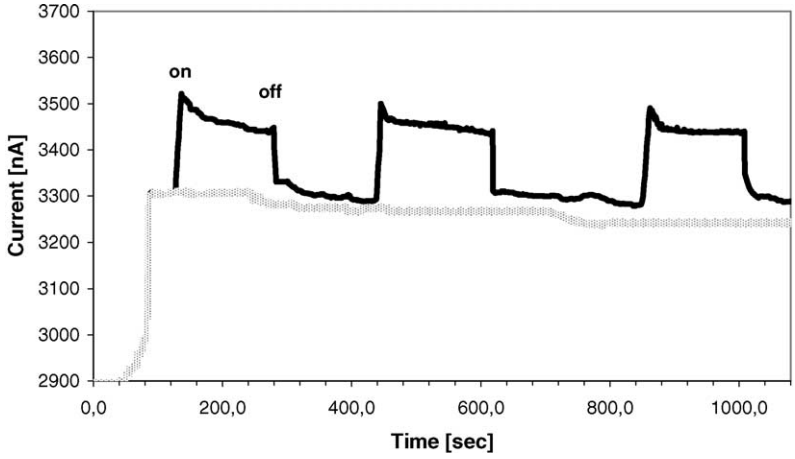

Fig. 5. Photocurrent response using FDH/semiconductor NCs/gold sensor: (on) illumination; (off) dark. Fixed concentration of injected formaldehyde: 1 μg mL^{-1}. Black curve: experiment with enzyme-based membrane; grey dotted curve: blank experiment with nude activated membrane.

Table 1
Selectivity of the amperometric detection of formaldehyde using FDH/semiconductor NCs/gold sensor

Interferent injected amount: 1.0 ppm	Possible applications	Relative peak height in the presence of H_2CO (%)	Relative peak height in the absence of H_2CO (%)
Without		100.0 ± 1.1 ($n = 6$)	
L(+)-Ascorbic acid	Beverage and pharmaceutical	89.0 ± 1.7 ($n = 6$)	-3.8 ± 0.4 ($n = 11$)
Glucose	Food and medicine	99.7 ± 0.9 ($n = 10$)	0.0 ± 0.0 ($n = 6$)
Glycine	Food	96.5 ± 0.9 ($n = 5$)	0.0 ± 0.0 ($n = 5$)
Paracetamol	Pharmaceutical	98.4 ± 0.9 ($n = 5$)	0.0 ± 0.0 ($n = 5$)
Methanol	Food and beverage	99.4 ± 0.8 ($n = 6$)	0.0 ± 0.0 ($n = 6$)
Cysteine	Chemicals and food	57.1 ± 1.6 ($n = 6$)	-8.5 ± 1.7 ($n = 6$)
Sulphite	Chemicals	41.8 ± 0.9 ($n = 6$)	-5.1 ± 0.8 ($n = 6$)

H_2CO concentration: 1.0 $\mu g\,mL^{-1}$ (ppm); amount of the injected interferent: 1.0 $\mu g\,mL^{-1}$ (ppm); flow rate: 0.3 $mL\,min^{-1}$; level of confidence, α: 0.05; n: number of repeated measurements.

place, several compounds show similarities with formaldehyde, originating false positive detection. To verify the selectivity of the proposed indicator electrode, the influence of different electrochemically active compounds in commonly used aqueous solutions was investigated both in the presence and the absence of formaldehyde. To this purpose 1.0 $\mu g\,mL^{-1}$ of formaldehyde solution was mixed continuously with 1.0 $\mu g\,mL^{-1}$ of interferent in PB solutions, under flow conditions, at a flow rate ratio of 1:1 just before the injection valve. After each injection of the mixed formaldehyde/interferent solution, the same amount of pure formaldehyde was injected: the measured peak heights were related to the peak height measured for formaldehyde in the absence of any interferent. Table 1 summarises the results. Among the selected components, glycine showed partial interference in the presence of formaldehyde, whilst significant interferences both in the presence and absence of formaldehyde was observed for ascorbic acid, cysteine and sulphite, when they were present in the ppm concentration regime. Anodic interferences and, after a longer contact, a significant loss of sensitivity of the current response indicate a possible poisoning of the electrode surface, especially for cysteine and sulphite, probably due to the specific interaction between the sulphur atoms and the golden support of CdS nanoparticles. By the way, it should be noted that paracetamol, glucose, glycine and methanol did not show any significant effect. These results appear very interesting, especially for avoiding false positive of formaldehyde detection in the presence of methanol; in fact methanol is used as a stabiliser for formaldehyde solution and is often found in mixed vapours and matrices containing H_2CO [65].

4. Conclusions

Photocurrent time course onto a gold electrode modified with CdS nanoclusters immobilized by the well known *self-assembling* techniques, in the presence of formaldehyde and enzyme membrane were recorded under illumination. The photo-amperometric response of the combined system, consisting in coupling to the sensor the covalently bonded enzyme (FDH) onto an Immunodyne™ membrane, was generally increased in comparison to a similarly designed system [48]. Current increases confirmed the role of the CdS nanoparticles as charge carrier in the enzymatic oxidation of formaldehyde instead of using the NAD^+ cofactor. In order to ascertain the sensing mechanism, blank experiments were made. No current was observed in the absence of enzyme onto the nude membrane after the substrate injection, once the system is photo-activated, this excluding the possibility to oxidize formaldehyde in the absence of enzyme.

Experimental conditions were optimized in sensitivity and reproducibility, by using a homemade three electrodes cell, varying the flow rate and the applied voltage. A detection limit of 41 ng mL^{-1} was calculated with a linear response in a relatively small range for the formaldehyde detection. The sensor stability after several cycles of photo-activation processes was evaluated in order to verify the optimization of the enzyme immobilization procedure onto the membrane. A relatively considerable medium term stability of the sensor was reached under flow conditions, confirming the possibility to keep the enzyme properties by means of chemical immobilization. The selectivity of the sensor was measured with respect to some interferent substances, giving encouraging results.

The study tested the effectiveness of CdS nanoparticles to replace the cofactor couple NAD^+/NADH as charge transfer in the enzymatic oxidation of formaldehyde, for future perspectives in novel electrochemical recognition biosensors.

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