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A hyaluronic acid dispersed carbon nanotube electrode used for a mediatorless NADH sensing and biosensing

Jaroslav Filip[a], Jana Šefčovičová[a], Peter Tomčík[b,c], Peter Gemeiner[a], Jan Tkac[a,*]

[a] Department of Glycobiotechnology, Center for Glycomics, Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, Bratislava, SK-845 38, Slovakia
[b] Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Radlinského 9, Bratislava, SK-812 37 Slovakia
[c] Department of Chemistry, Faculty of Education, The Catholic University in Ružomberok, Hrabovská cesta 1, Ružomberok, SK-034 01, Slovakia

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ABSTRACT

A biocompatible nanocomposite consisting of single-walled carbon nanotubes (CNTs) dispersed in a hyaluronic acid (HA) was investigated as a sensing platform for a mediatorless electrochemical detection of NADH. The device was characterised by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and extensively by electrochemistry. CNT–HA bionanocomposite showed more reversible electrochemistry, higher short-term stability of NADH sensing and higher selectivity of NADH detection compared to frequently used CNT–CHI (chitosan) modified GCE. Finally the performance of the sensor modified by CNT–HA was tested in a batch and flow injection analysis (FIA) mode of operation with basic characteristics revealed. The NADH sensor exhibits a good long-term operational stability (95% of the original sensitivity after 22 h of continuous operation). Subsequently a D-sorbitol biosensor based on such a nanoscale built interface was prepared and characterised with a D-sorbitol dehydrogenase used as a biocatalyst.

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1. Introduction

NAD(P)H is a cofactor of more than 500 enzymes and its regeneration is pivotal for many practical applications (e.g. biosynthesis, preparation of biosensors and biofuel cells) [1]. Electrochemical oxidation of a NAD(P)H occurs at high overvoltage (~1 V) and is accompanied by a cofactor dimerisation and subsequently by fouling of the electrode surface [2,3]. Chemically modified electrodes were used to solve all these problems [2] with phenoxazines and phenazines introduced by Gorton in 80s still of frequent use [4]. Another successfully used mediator systems for NADH oxidation are from the group of quinones and their derivatives [5–7], but other redox shuttles including electropolymerised ones can be used, as well [1–3].

A new era of NADH detection using advanced nanomaterial started by Wang in 2002 using carbon nanotubes [8]. Various forms of nanostructures are used for mediatorless/reagentless NADH detection including mesoporous carbon, nanoporous metals and graphene sheets at reduced overvoltage (from +0.5 to +0.8 V *vs.* NHE) with detection limit in low μM range [9–11], but CNTs are the most frequently used [1,3,12–14]. CNTs have beneficial redox properties due to presence of edge plane sites and defects as revealed by Compton's group [15]. Moreover, CNTs are the matrix with well known exceptional resistance towards surface passivation (as a result of NADH oxidation) [16–18]. Pristine CNTs are very difficult to dissolve/disperse in most organic or aqueous solutions [19] and they have to be treated by a variety of modification protocols [20–22]. A dispersing agent should provide biocompatibility and functionalities available for grafting of biocatalysts and/or docking of redox shuttles. Biopolymers can fulfil all these criteria, what can facilitate further development in many fields with improved performance over existing technologies [19,23].

Polysaccharide-based biopolymers (e.g. hyaluronic acid – HA and chitosan – CHI) are of high interest for various applications due to their ease of processing into useful structures [24]. CNTs were successfully blended with these biopolymers with applications in the area of tissue engineering with enhanced mechanical support [23–26]. Although CHI is often used to disperse CNTs with subsequent preparation of electrochemical (bio)sensors [27,28], recent studies suggest HA can be efficiently used for the same purpose as judged from excellent conductivity of the nanocomposite and remarkable dispersivity of HA towards CNTs [23,29].

HA occurring in a variety of sizes has a non-branched structure consisting of repeating units of two saccharides with an array of different regulatory/structural functions with involvement in the body's alarm system [30]. HA has been used as a dispersing agent to solubilise CNTs with the focus on preparation of biofibers to be used in tissue engineering and for growth of fibroblast cells

* Corresponding author. Tel.: +421 2 5941 0263; fax: +421 2 5941 0222.
E-mail address: Jan.Tkac@savba.sk (J. Tkac).

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[23,29,31,32]. In this report HA dispersed CNTs are used for preparation of an amperometric NADH sensor with improved operational stability and overall performance when compared to GCE modified by CNT dispersion in CHI or DMF.

2. Experimental procedures

2.1. Reagents

Single walled CNTs (OD = 1.1 nm, L = 0.5–100 μm, >90% purity), Nafion (20% solution in low molecular weight alcohols), chitosan (CHI, degree of deacetylation of 85%), hyaluronic acid (HA from *Streptococcus equi*), ascorbic acid (AA), dopamine (DOP), dimethylformamide (DMF) and D-sorbitol dehydrogenase (SDH from sheep liver) were purchased from Sigma–Aldrich (St. Louis, USA). Reduced β-nicotinamide adenine dinucleotide (NADH) and its oxidised form (NAD^+) were purchased from Sorachim (Paris, France). 50 mM phosphate buffer of pH 8.0 (PB) was prepared from KH_2PO_4 and K_2HPO_4 (Mikrochem, Pezinok, Slovakia).

2.2. Instrumentation

The FIA system (FIAlab Instruments Inc., Bellevue, USA) consisting of peristaltic pumps (Watson-Marlow, Houston, USA), sampling/injection valves (Vici, Houston, USA) and a flow cell (7 μl, Cypress System, Lawrence, USA) was running with an injection loop of 20 μl and a flow rate of 0.21 ml min^{-1} in PB. The batch mode of operation was performed in a glass vessel in 10 ml of PB using a stirrer (250 rpm). An utility designed in Labview (National Instruments, Austin, USA) was used for control and data acquisition [33].

For electrochemical measurements a potentiostat Autolab PGSTAT 128N and 302N (Ecochemie, Utrecht, Netherland) was used with glassy carbon electrode (GCE) as a working one provided by BAS (batch and CV assays with d = 3 mm) or Cypress System (FIA with d = 1 mm). An Ag/AgCl reference electrode was used in FIA, while Hg/Hg_2SO_4 and Ag/AgCl reference ones were used for batch and CV experiments. A potential of +400 mV *vs.* Ag/AgCl electrode (−50 mV *vs.* Hg/Hg_2SO_4) was used in batch and FIA measurements.

Scanning electron microscope SEM ZEISS EVO 40 was accompanied with an EDX BRUKER detector (Carl Zeiss, Germany). FTIR spectra were measured on spectrometer Nicolet 6700 (Thermo Fisher Scientific, USA) with DTGS detector and Omnic 8.0 software. Spectra were collected from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} with 128 scans averaged with measurements in a solid state.

2.3. Procedures

Dispersions of CNTs were prepared from 1 mg of CNTs in 1 ml of 0.1% HA (solution in DW); in 1 ml of 0.1% CHI (solution in 1% acetic acid) or in 1 ml of DMF in an ultrasonication bath (Bandelin DT 102 H, Bandelin electronics, Berlin, Germany) at 25 °C for 20 min.

All GC electrodes were polished using 0.3 μm alumina/diamond slurry (Struers A/S, Ballerup, Denmark) and sonicated in distilled water for 30 s. A GCE covered by a single layer of CNT dispersion (5 μl) was used for CV measurements. A GCE for batch experiments was modified by casting of 5 μl of CNT dispersion, allowed to dry, subsequently covered by 10 μl of 0.5% Nafion in PB and left to dry prior use. A CNT layer on GCE for FIA configuration was formed by pippetting of 2 μl of CNT dispersion, subsequently covered by 4 μl of 0.5% Nafion in PB in three steps (30 min drying period). In an interference study the CNT layer was covered in two steps by 5 μl of either Nafion (0.5% solution in PB) or chitosan (CHI, 0.1% solution in 0.3% acetic acid) or the CNT layer was used without any protective outer membrane present. When the effect of HA matrix itself on the oxidation of NADH was investigated, GCE was covered by 5 μl of 0.1% HA (solution in DW).

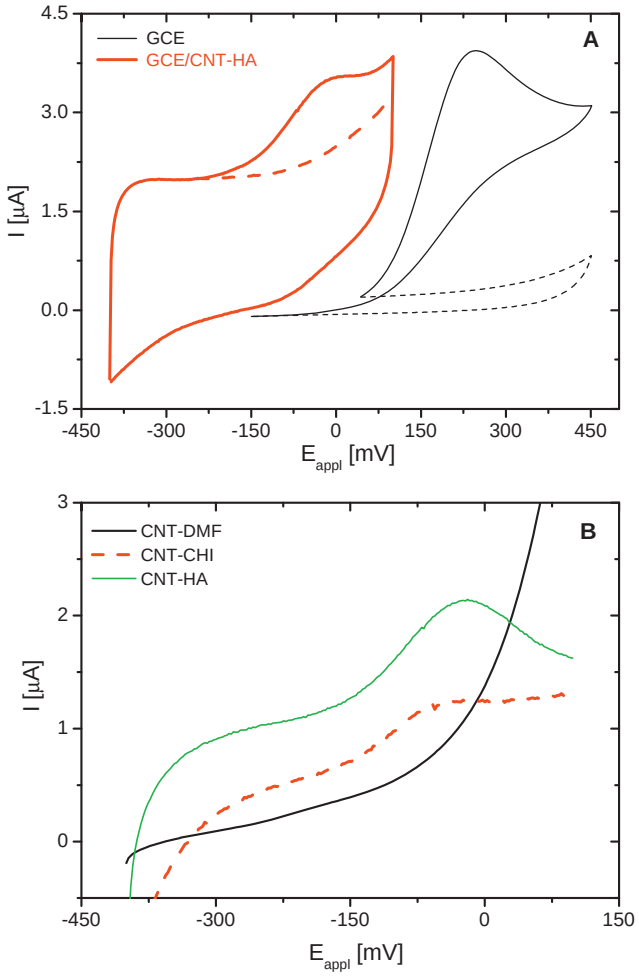

Fig. 1. (A) Electrochemistry of 1 mM NADH in 50 mM PB 8.0 on a bare GCE and GCE modified by a CNT–HA dispersion. Only a relevant part of a background CV is shown for a better clarity. (B) Comparison of a LSV obtained in 1 mM NADH subtracted from a background LSV for CNT–HA, CNT–DMF or CNT–CHI dispersions, respectively. Experiments were run at a sweep rate of 50 mV s^{-1} using Hg/Hg_2SO_4 reference electrode.

The biosensor device was built up with a CNT–HA layer (5 μl) cast as the first layer followed by a formation of an enzyme layer (3 μl of 8% SDH solution, if not mentioned otherwise) with the final layer cast from 0.5% Nafion in PB in 3 steps (if not mentioned otherwise). This procedure is referred as a layer by layer deposition in the following text. Alternatively, a bionanocomposite layer was deposited on a GCE surface from a blend of CNT–HA (5 μl) and 8% SDH (3 μl) deposited in one step and subsequently covered by a Nafion layer (5 μl of 0.5% solution in PB) cast in 3 steps and referred in the text as a blend deposition.

3. Results and discussion

3.1. Characterisation of the bionanohybrid layer

Electrochemical characterisation was performed by cyclic voltammetry (CV) and linear sweep voltammetry (LSV). A NADH detection on a CNT nanocomposite layer can be seen in Fig. 1A with an anodic peak potential of NADH oxidation at −26 mV (*vs.* Hg/Hg_2SO_4), while an anodic peak potential of NADH on a bare GCE was observed at +229 mV (*vs.* Hg/Hg_2SO_4). Electrochemical behaviour of NADH on a HA modified GCE electrode had the same pattern as on unmodified GCE (data not shown), suggesting only CNTs have electrocatalytic effect with lowering of the overpoten-

Table 1
Summary of parameters obtained from electrochemical investigation of redox behaviour of two redox probes on GCE modified by three CNT dispersions.

	$[Fe(CN)_6]^{3-}$						$[Ru(NH_3)_6]^{3+}$					
	E_{pc} (mV)	E_{pa} (mV)	ΔE_p (mV)	$E^{\circ\prime}$ (mV)	I_{pc} (μA)	I_{pa} (μA)	E_{pc} (mV)	E_{pa} (mV)	ΔE_p (μA)	$E^{\circ\prime}$ (mV)	I_{pc} (μA)	I_{pa} (μA)
CNT–DMF	288	195	93	242	6.4	6.1	−131	−195	64	−163	8.2	5.6
CNT–HA	273	208	65	241	8.8	8.5	−134	−197	63	−166	9.0	6.7
CNT–CHI	276	205	71	241	7.9	8.4	−134	−202	68	−168	8.6	5.0

E_{pc}, E_{pa} – potential of cathodic peak or anodic peak, respectively; ΔE_p – peak separation; $E^{\circ\prime}$ – formal potential defined as $(E_{pa}+E_{pc})/2$; I_{pc}, I_{pa} – current of cathodic peak or anodic peak, respectively. Experiment was performed with an Ag/AgCl reference electrode.

tial needed to detect NADH. Thus, HA is electrochemically inactive matrix with a predominant role as a dispersing agent for CNTs. Even though much larger oxidation peak can be seen on an unmodified GCE electrode compared to GCE modified by CNT–HA, the applied potential for NADH oxidation is high on unmodified GCE with subsequent fouling of the electrode by oxidation products of the analyte [1–3]. Such passivation of the electrode surface negatively affects overall performance of NADH sensing.

Furthermore, a detection of NADH on a CNT–HA nanocomposite film was compared to detection of NADH on a CNT–CHI layer, frequently used for sensing and biosensing [34,35] (Fig. 1B). The anodic peak on a CNT–HA layer is much larger (2.0 μC) compared to the CNT–CHI layer (0.6 μC) indicating better redox charge transfer of a CNT–HA nanocomposite towards NADH. An anodic peak for NADH on GCE modified by CNT–DMF dispersion was not observed and this is why the area under the peak could not be read (Fig. 1B). This observation is consistent with chronoamperometric detection of 1 mM NADH, which was the most sensitive on GCE modified by CNT–HA, followed by GCE/CNT–CHI (approx. 32% of the signal on CNT–HA) and finally by GCE/CNT–DMF done at the same applied potential of +400 mV *vs.* Ag/AgCl electrode (data not shown).

GCE modified by CNTs dispersions prepared using three dispersing agents (DMF, CHI and HA) was electrochemically probed using a positively charged mediator $[Ru(NH_3)_6]^{3+}$ and a negatively charged one $[Fe(CN)_6]^{3-}$, as well (Fig. 2). This study confirms electrochemistry of both redox probes is the most favourable on GCE modified by CNT–HA, followed by GCE modified by CNT–CHI and finally by GCE modified by CNT–DMF. This conclusion can be drawn from peak separation and peak currents summarised in Table 1. Peak separation for $[Ru(NH_3)_6]^{3+}$ of 63 mV and for $[Fe(CN)_6]^{3-}$ of 65 mV on GCE modified by CNT–HA is close to the theoretical value of 59 mV for one electron fully reversible redox process, showing excellent redox reversibility of the nanocomposite. Redox behaviour of both redox probes was less reversible (e.g. ΔE_p of 68 mV or 71 mV, respectively) on GCE modified by CNT–CHI (Table 1).

Favourable dispersive properties of HA towards CNT can be judged from FTIR spectra (Fig. 3), revealing quite large shift of the main peaks of HA: 1612 → 1597 (an asymmetric C=O band from COO^-), 1410 → 1404 (a symmetric C=O band from COO^-) and 1046 → 1032 cm^{-1} (C–OH band of a saccharidic structure), when HA was integrated with CNTs [36]. These large shifts suggest all three major bonds of HA exist in a different environment after being integrated with CNTs. A similar shift of the frequency towards lower frequencies of various bonds within a biopolymer (chitosan) after being integrated with nanostructures was noticed [37,38].

An ability of HA to efficiently make dispersions of CNTs was checked by SEM. When CNTs were cast on the conductive surface from distilled water, CNTs can be clearly seen (Fig. 4B), while SEM of the nanocomposite layer shows a uniform and flat layer

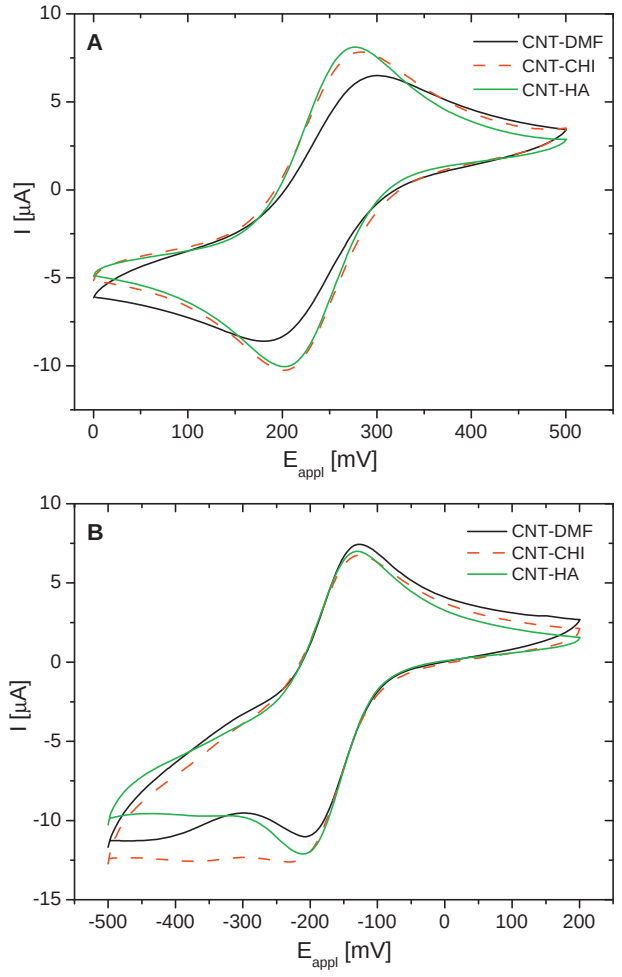

Fig. 2. (A) Electrochemistry of 1 mM $[Fe(CN)_6]^{3-}$ on GCE modified by three different CNT dispersions (e.g. CNT–DMF, CNT–CHI and CNT–HA). (B) Electrochemistry of 1 mM $[Ru(NH_3)_6]^{3+}$ on GCE modified by the same CNT dispersions. Experiments were run at a sweep rate of 50 mV s^{-1} using Ag/AgCl reference electrode in 50 mM PB 8.0 with 0.1 M KCl.

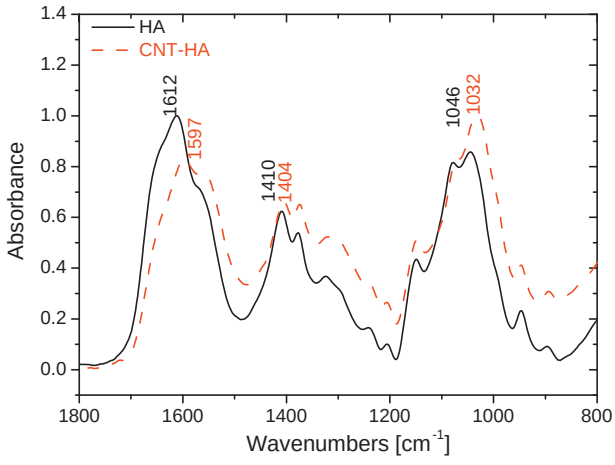

Fig. 3. FTIR spectra of a HA layer or a bionanohybrid CNT–HA layer, respectively.

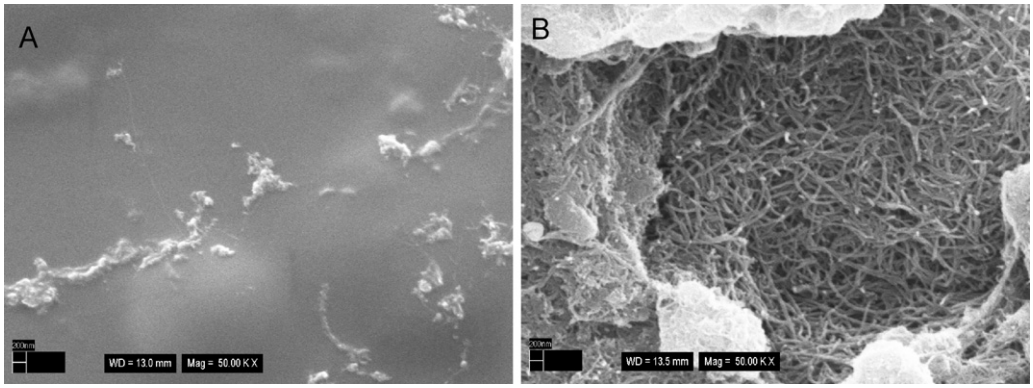

Fig. 4. SEM image of (A) a CNT–HA bionanohybrid layer and (B) a CNT layer.

of the bionanocomposite with CNTs integrated within the matrix (Fig. 4A).

3.2. Basic properties of the sensor device

A CNT dispersion in HA with subsequent GCE modification was used to investigate properties of NADH sensing evaluated in a batch and FIA operation mode.

In a batch mode, measurement was carried out until a steady-state current after each NADH addition was reached with a sensitivity of $(6.86 \pm 0.03)\,\mu A\,mM^{-1}\,cm^{-2}$ ($R^2 = 0.999$). A typical sensitivity of NADH sensor devices based on electrodes modified by various types of mediators and transducing surfaces is in the range from tens to hundreds of $\mu A\,mM^{-1}\,cm^{-2}$ [1,3,5–7,39], but in the case of NADH sensor devices based on CNT modified electrodes without any redox shuttle the sensitivity is in the range from 3 to 83 $\mu A\,mM^{-1}\,cm^{-2}$ [39–42] and only occasionally in the range of hundreds of $\mu A\,mM^{-1}\,cm^{-2}$ [43].

A response time of the presented NADH sensor device was 19 s (90% of steady state current) (Fig. 5). A typical response time of NADH sensors is in the range of few seconds [1,3,5–7] and in the case of CNT-based NADH sensors it can be in the range from 3 to 50 s [8,42–45]. Detection limit of the NADH device for its analyte was higher (e.g. 63 μM, S/N = 3) compared to published mediator integrated systems with the value in low micromolar range [1,3,5–7,39,46] and similar to other CNT-based NADH devices without integration of a redox shuttle (e.g. from 2 to 40 μM) [42,44,47–49]. Detection of NADH exhibited high signal repeatability with an average RSD of 3.6% (variation range 1.0-5.0%, $n = 3$) and good batch to batch reproducibility of preparation of CNT dispersions and modified GCE (9.2%, $n = 3$).

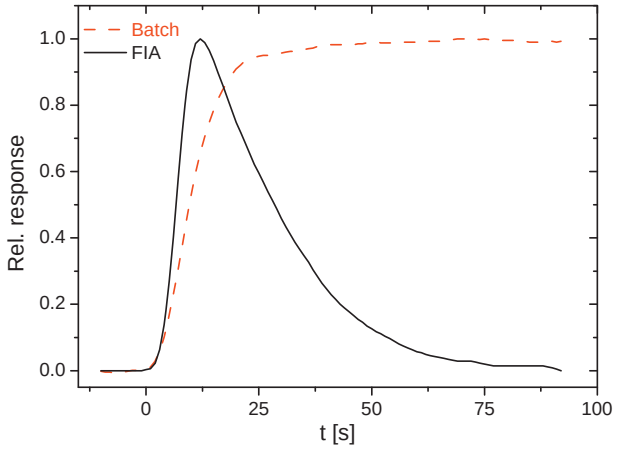

Fig. 5. A normalised response of the NADH sensor based on GCE modified by CNT–HA towards 0.5 mM NADH in a batch mode (red dashed line) or towards 3 mM NADH in a FIA mode (black solid line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

For many practical applications of sensor devices, there is a strong need for higher throughput and automation of the assay procedure, what can be performed by integration of the sensor device within flow injection analysis (FIA) system introduced in 70s [50]. This concept of analysis is gaining popularity in the area of sensor and biosensor technology due to some advantages [51] and that is why a CNT modified GCE electrode was integrated within a flow through cell. An evaluation revealed the sensor sensitivity integrated into FIA of $(7.13 \pm 0.13)\,\mu A\,mM^{-1}\,cm^{-2}$ ($R^2 = 0.999$, $n = 3$). Moreover response time of the sensor in the FIA mode of operation was 59 s (Fig. 5) with a high throughput rate of 61 h^{-1} and detection limit of 130 μM. FIA gave quite repeatable results with an average RSD of determination of 6.7% (variation range 1.5–10.3%, $n = 4$).

It is quite interesting to compare performance of NADH sensor device working in batch or FIA mode of operation, respectively. The sensitivity in the batch mode is 6.9 $\mu A\,mM^{-1}\,cm^{-2}$, while in the FIA mode is even higher with the value of 7.1 $\mu A\,mM^{-1}\,cm^{-2}$ with the ratio $S_{FIA}/S_{batch} = 1.03$. This value is much higher compared to two other papers published with the ratio of 0.14 [51] or 0.22 [43], suggesting the flow cell used here provides high performance of NADH detection.

A short term stability of the device based on GCE modified by CNT–HA was compared to the device prepared by casting CNT–CHI on a GCE electrode. The study performed in a batch mode of operation showed an excellent short term stability of NADH detection on GCE modified by CNT–HA with the final relative sensor response unaffected for 56 min (Fig. 6A). The sensitivity of NADH device based on GCE modified by CNT–CHI nanocomposite dropped sharply to 74% of the initial response within the same time frame (56 min). This decrease of NADH sensing ability on CNT–CHI surface is in a good agreement with a sharp decay of the NADH sensing on other CNT-based NADH devices with a decrease of the signal in the range from 4 to 24% just in 1 h [8,41,42,52]. The reason for an excellent short-term stability of NADH device based on GCE modified by CNT–HA nanocomposite is not known at the present.

A long-term stability of the CNT modified interface to detect NADH was investigated by continuous operation of the device integrated in a FIA cell by injection of 3 mM NADH with frequency of 4 h^{-1}. The initial sensitivity of $(7.00 \pm 0.13)\,\mu A\,mM^{-1}\,cm^{-2}$ decreased to $(5.35 \pm 0.10)\,\mu A\,mM^{-1}\,cm^{-2}$ in 22 h, but when a freshly prepared NADH solution was injected a higher sensitivity of $(6.62 \pm 0.15)\,\mu A\,mM^{-1}\,cm^{-2}$ (95% of the initial response) was revealed (Fig. 6B). This short-term and long-term stability of the sensor device is crucial for subsequent development of biosensors or biofuel cells based on this bionanocomposite.

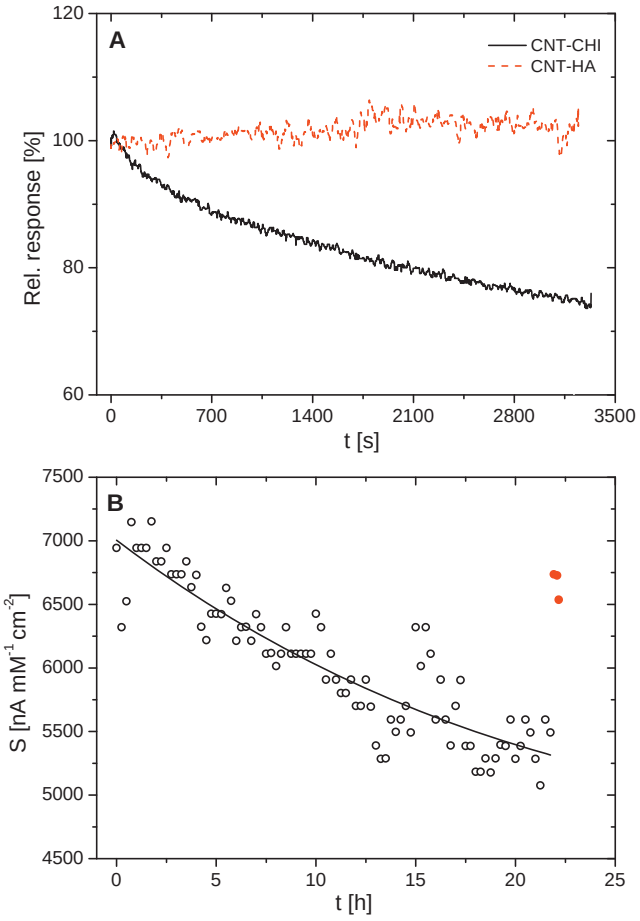

Fig. 6. (A) A short term operational stability of the device prepared by modification of GCE either by CNT–CHI or CNT–HA nanocomposite towards 1 mM NADH investigated in a batch mode of operation. (B) A long-term operational stability of the NADH sensor device based on GCE modified by CNT–HA towards 3 mM NADH showing FIA response during its continuous use. A FIA response shown at time interval of 22 h (full red circles) is due to injection of a freshly prepared 3 mM NADH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

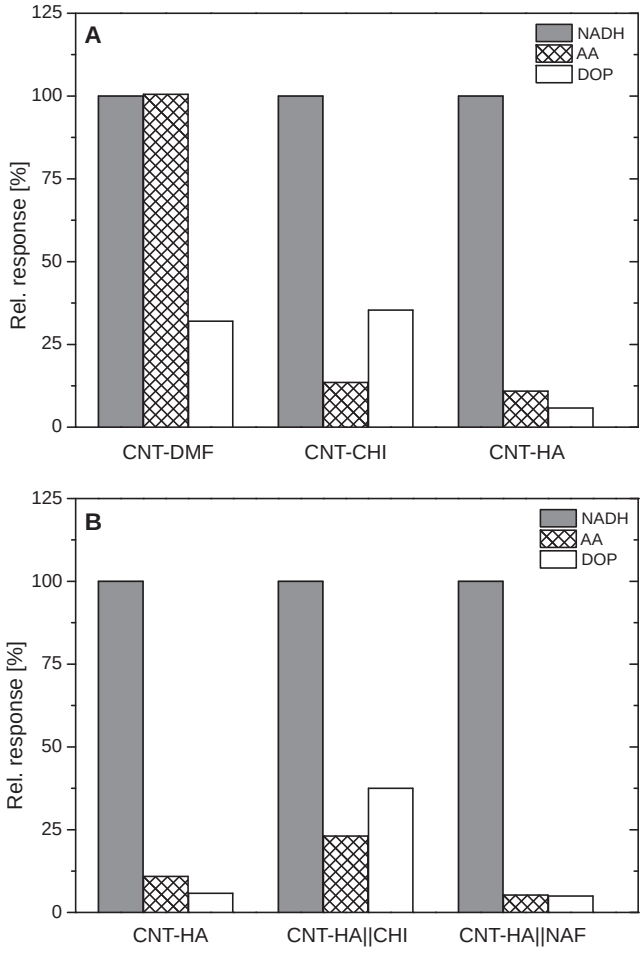

Fig. 7. (A) An interference study of the NADH sensors prepared by modification of GCE using three different CNT dispersions (CNT–DMF, CNT–CHI and CNT–HA) investigated with two common electrochemical interfering compounds AA (ascorbic acid) and DOP (dopamine). (B) The effect of an outer layer (e.g. chitosan–CHI or Nafion–NAF) on the selectivity performance of NADH device in the presence of interfering compounds. Electrochemical investigation was carried out using 1 mM NADH, 0.05 mM AA and 0.02 mM DOP (both compounds present in physiologically relevant concentrations) [44]. Current output towards 1 mM NADH was set to 100% for each modified electrode.

3.3. An interference study

In order to prove a beneficial role of HA matrix on the selectivity performance of the NADH sensor device a comparison of three nanointerfaces prepared by casting of three dispersions of CNTs either in HA, CHI or DMF was carried out. DMF as a dispersant was chosen because of its frequent use in preparing CNT dispersions [28,53] and because after evaporation there is no matrix left and thus such a CNT layer can be considered as a reference layer. CHI-CNT was implemented in the study because this bionanocomposite is of frequent used in various devices integrated with CNTs. Two electrochemically active compounds were used in the study e.g. ascorbic acid (AA) and dopamine (DOP), which are present in the human serum samples in concentration of 0.05 mM or 0.02 mM, respectively [54].

A CNT layer cast from DMF was not able to discriminate between NADH and AA (101% of NADH signal) with a large current observed in the presence of DOP (32% of NADH signal) (Fig. 7A). A sensor device based on GCE modified by CNT–CHI nanocomposite showed better selectivity of NADH detection in the presence of AA, but with approximately the same level of interference coming from DOP (when compared to GCE/CNT–DMF). A CNT–HA based sensor device was able to detect NADH more selectively compared to AA (11% of NADH signal) or DOP (6% of NADH signal), respectively (Fig. 7A). This study confirms the importance of HA matrix on an enhanced performance of NADH detection in the presence of common interferents.

The effect of two additional outer layers (CHI or NAF) on the selectivity of NADH detection was investigated and compared to the sensor without outer layer. A CHI outer layer completely reverted a positive effect of HA matrix on enhanced selectivity of NADH detection. It is interesting that after deposition of CHI outer layer (e.g. sensor configuration of CNT–HA||CHI, Fig. 7B) the selectivity profile of the device is similar to the device prepared by deposition of CNT–CHI dispersion on GCE (e.g. sensor configuration of CNT–CHI, Fig. 7A). The possible explanation is an electrostatic interaction between these two polymers (HA and CHI), thus, shielding a negative charge of –COOH functionalities within HA matrix. A small beneficial role of the outer Nafion layer present on the CNT–HA nanocomposite was observed with a decrease of the sensor sensitivity towards AA from 11% to 5% of the NADH signal, when compared to the sensor surface without any outer layer (Fig. 7B). Only a minor change of the sensor sensitivity was observed for DOP with 6% of NADH signal observed on CNT–HA surface compared to 5% of NADH signal seen on CNT–HA surface covered by a Nafion outer layer (Fig. 7B). This really proves HA matrix cast from 0.1%

solution had almost as good properties to exclude interfering compounds as a Nafion layer cast from a much more concentrated 0.5% solution in two steps (this means 10× higher amount of Nafion matrix by weight compared to HA matrix on the GCE surface).

3.4. A biosensor device

Three different modes of preparation of the biosensor device were optimised including concentration of the enzyme, number of Nafion layers used as an outer protective membrane and the way the bionanocomposite is prepared (e.g. layer by layer modification or a modification by a blend of CNT–HA and SDH).

Initially, the influence of the thickness of the outer Nafion layer on the biosensor performance with 4% SDH used in the immobilisation step was optimised. When a single layer of Nafion was cast on the CNT–HA layer modified by SDH a low sensitivity was observed and the layer came apart from the GCE surface after the modified electrode was rinsed by a PB. Two layers of Nafion provided more stable biosensor response with sensitivity of detection of 3.56 $\mu A\,mM^{-1}\,cm^{-2}$. Three layers of Nafion were able to keep slightly larger amount of the enzyme on the electrode surface with sensitivity of 3.96 $\mu A\,mM^{-1}\,cm^{-2}$, detection limit of 29 μM (S/N = 3) and a response time of 38 s (90% of steady-state current response).

Secondly, the effect of the enzyme concentration (1%, 2%, 4% and 8%) cast from 3 μl of the solution on the CNT–HA interface on the biosensor performance was evaluated. The highest sensitivity (6.02 $\mu A\,mM^{-1}\,cm^{-2}$) was observed with the biosensor device with an enzyme immobilised from 8% solution. Moreover, other operational characteristics such as a detection limit of 18 μM (S/N = 3) and a response time of 53 s (90% of steady-state current response) were revealed, as well.

Thirdly, a comparison between two approaches used to prepare a bionanocomposite layer was done e.g. a layer by layer concept *vs.* deposition of a blend of CNT–HA and SDH in a single step. Careful evaluation of the results revealed a layer by layer formation of a bionanocomposite was more sensitive (7.19 ± 1.03) $\mu A\,mM^{-1}\,cm^{-2}$ compared to deposition of a blend of CNT-HA and SDH (6.59 ± 0.81) $\mu A\,mM^{-1}\,cm^{-2}$, when 3 independent electrodes were prepared for both approaches. The value of sensitivity of the biosensor observed here is well within the range for mediator based biosensor systems (e.g. from 0.09 to 80 $\mu A\,mM^{-1}\,cm^{-2}$) [5–7,44,51,52] or for CNT-based dehydrogenase biosensors without the redox shuttle present (1.8–33 $\mu A\,mM^{-1}\,cm^{-2}$) [35,39,41,43,55].

Detection limit and response time was similar in both cases e.g. 18 μM and 53 s (layer by layer deposition); or 16 μM and 45 s (deposition of a blend) and both values are within published results e.g. a response time from 3 to 60 s and a detection limit from 1 to 100 μM [5–7,33,35,41,43,44,52]. Response time was much better than for other D-sorbitol biosensors published (60–300 s) [56,57]. A calibration of the biosensor for D-sorbitol prepared by both methods of the bionanocomposite deposition e.g. layer by layer and blend deposition is shown in Fig. 8.

It is interesting to compare performance of dehydrogenase-based biosensor to the performance of NADH-based sensor for a particular modification of the electrode surface. In our case only a minor decrease of the sensitivity of the biosensor (6.6 $\mu A\,mM^{-1}\,cm^{-2}$) compared to NADH sensor (6.9 $\mu A\,mM^{-1}\,cm^{-2}$) was observed with a ratio $S_{biosensor}/S_{NADH}$ of 0.96 underlining an excellent integration of the enzyme within a transducing surface. The values of this ratio available in the literature are far worse being within range from 0.0005 to 0.093 [6,7,39,41,43,51,52,58] with only 3 reports showing higher values e.g. from 0.52 to 0.63 [5,44,59].

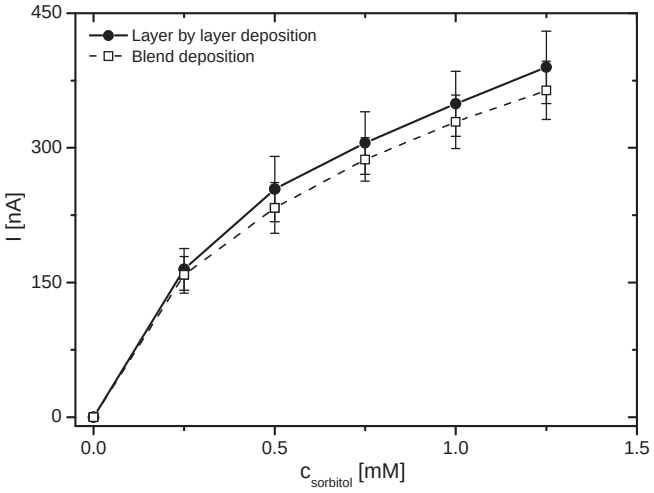

Fig. 8. A calibration curve of the D-sorbitol biosensor device based on different way the bionanocomposite is deposited on the GCE.

4. Conclusions

CNT–HA nanocomposite layer was characterised by SEM and FTIR showing strong interaction between **HA and CNTs necessary for preparing biocompatible dispersions. Moreover, a CNT–HA layer showed better redox charge transfer compared to frequently used CNT–CHI bionanocomposite or CNT cast from DMF as judged from extensive electrochemical investigation using two redox probes and NADH. Much higher short-term operational stability and selectivity of CNT–HA based device compared to device based on CNT–CHI nanocomposite is another beneficial feature of this novel NADH sensor. Finally, two modes of operation e.g. batch *vs.* FIA were compared with advantages and trade-off discussed. Long-term stability of a CNT modified interface was very good, a feature important for their integration into biofuel cells or biosensor devices. Finally, a biosensor with SDH as a biocatalyst was prepared with some steps of the biosensor preparation optimised. Basic operational parameters of the biosensor device were revealed.

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