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RESEARCH PAPER

Reagentless D-sorbitol biosensor based on D-sorbitol dehydrogenase immobilized in a sol–gel carbon nanotubes–poly(methylene green) composite

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Abstract A reagentless D-sorbitol biosensor based on NAD-dependent D-sorbitol dehydrogenase (DSDH) immobilized in a sol–gel carbon nanotubes–poly(methylene green) composite has been developed. It was prepared by durably immobilizing the NAD^+ cofactor with DSDH in a sol–gel thin film on the surface of carbon nanotubes functionalized with poly(methylene green). This device enables selective determination of D-sorbitol at 0.2 V with a sensitivity of $8.7\,\mu A\,mmol^{-1}\,L\,cm^{-2}$ and a detection limit of 0.11 $mmol\,L^{-1}$. Moreover, this biosensor has excellent operational stability upon continuous use in hydrodynamic conditions.

Keywords Carbon nanotubes · Sol–gel · Poly(methylene green) · Cofactor · Dehydrogenase · Reagentless biosensor

Introduction

More than 300 dehydrogenases, which catalyze the redox transformation of a large variety of substrates, have been found useful in analytical [1], preparative [2], or energy-related [3] applications. Nowadays, there is much interest in the development of electrochemical sensors for use in simple procedures, ideally operating under reagentless conditions. This can be achieved, for example, by direct electron transfer (DET) [4] from the protein to the electrode surface or, if DET is not applicable, by use of appropriate mediators [5]. Despite the long history of using dehydrogenases in the design of electrochemical biosensors [1, 6], there are few reports on the construction of dehydrogenase-based reagentless biosensors [7–14], because of several difficulties.

The first difficulty is immobilization of the NAD^+ cofactor on to the electrode surface. Several strategies have been described in the literature; these are essentially based on chemical grafting of the cofactor on to macromolecules, e.g. polyethyleneimine [15, 16], alginate [10], chitosan [11], poly(ethylene glycol) [17], or dextran [18], and their subsequent immobilization on the electrode surface by coating or entrapment in a host matrix. Recently, our group reported chemical attachment of NAD^+ to glycidoxypropylsilane in the course of sol–gel thin film deposition with concomitant protein encapsulation on the electrode surface [13]. This procedure enables durable immobilization of NAD^+ in the sol–gel matrix while maintaining good interaction between NAD^+ and the dehydrogenase.

The second difficulty can be ascribed to the electrochemistry of the NADH cofactor itself. Direct oxidation of NADH on a bare electrode usually requires high overpotential that can lead to enzymatically inactive forms of the cofactor and can be disturbed by interfering reactions occurring at the same potential, which is detrimental to analytical applications [1]. For this reason, it is necessary to catalyze the electrochemical oxidation of NADH, for example by use of a second protein [19], e.g. diaphorase in the presence of such mediators as ferrocene, an osmium complex, or vitamin K_3 [20, 21]. In previous work we used diaphorase to catalyze regeneration of the cofactor NAD^+

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covalently immobilized inside sol–gel films under reagentless conditions [13, 14]. This strategy works well, but there is still interest in avoiding the use of diaphorase (i.e., simplified electrochemical pathway, cheaper sensor preparation) and enabling direct regeneration of the cofactor by confining the mediator only to the electrode surface in a configuration likely to facilitate interfacial electron-transfer reactions.

Several mediators, for example quinones [22, 23], oxometalates [24], or phenazines [25] and phenoxazines [26, 27], have been proposed for cofactor regeneration. Traditionally, the mediators were directly adsorbed, electropolymerized, or covalently bound to the electrode surface [28–30]. In recent years, the use of carbon nanotubes (CNT) for mediator immobilization has attracted much attention [31, 32]. Several dyes, for example toluidine blue [33], meldola blue [34], and methylene green (MG) [35], have been immobilized by absorption on to CNT, resulting in remarkable improvement of electocatalysis of NADH oxidation. The stability of such devices can be improved by covalent bonding of dyes to CNT, as shown by Ohsaka et al. [36] for Nile blue covalently attached to the surface of functionalized CNT. Electropolymerization is another approach used to form stable films of mediators on electrode surfaces [28, 37]. In this context, electropolymerized methylene green on a glassy carbon electrode (GCE) surface has good electrocatalytic properties in the oxidation of NADH [38, 39]. Recently, this approach has been successfully extended to the functionalization of CNT-based bucky papers and carbon nanotube-modified electrodes for application to the electrocatalytic oxidation of NADH [40, 41].

In this work we developed a reagentless D-sorbitol biosensor, based on dehydrogenase and its cofactor, co-immobilized in a sol–gel of a carbon nanotubes–poly(methylene green) composite. Electrocatalytic oxidation of NADH by use of carbon nanotubes–poly(methylene green)-modified electrodes was first characterized with this cofactor in solution. It was then applied to regeneration of NAD^+ durably immobilized with D-sorbitol dehydrogenase in a thin sol–gel film. This proposed reagentless biosensor has since been used for detection of D-sorbitol in candy samples.

Experimental

Chemicals and reagents

D-Sorbitol dehydrogenase (DSDH) solutions (10 $mg mL^{-1}$, 100 units mg^{-1}) were prepared as described elsewhere [42]. β-Nicotinamide adenine dinucleotide (NAD^+; ~98 %, Sigma), D-sorbitol (98 %, Sigma), tetraethoxysilane (TEOS; 98 %, Alfa Aesar), methylene green (MG; Sigma), 3-glycidoxypropyltrimethoxysilane (GPS; 98 %, Aldrich), poly(ethyleneimine) (PEI; high molecular weight, water-free, Aldrich), chitosan (medium molecular weight, Aldrich), Tris(hydroxymethyl)aminomethane (Tris; 99 %, Sigma), HCl (36 %, Prolabo), and multi-walled carbon nanotubes (MWCNT, 95 %, Aldrich) were used without further purification. Tris–HCl (pH9.0) buffers were prepared by adding suitable amounts of HCl to 0.1 $mol L^{-1}$ Tris solutions. A 20 % (*w*/*w*) PEI solution was prepared by dissolving an appropriate amount of PEI in water, and the final pH was adjusted to 9.0 with HCl. All solutions were prepared with high-purity water (I + quality, Purelab, Elga).

Apparatus

Cyclic voltammetry (CV) and chronoamperometry experiments were performed by use of an Autolab PGSTAT-12 potentiostat (Eco Chemie) monitored by GPES (General Purpose Electrochemical System) software. Measurements were performed at room temperature in a three-electrode configuration comprising a modified glassy carbon working electrode, an Ag/AgCl (saturated KCl internal electrolyte) as reference electrode and a gold wire as counter electrode.

Preparation of modified electrodes

The electrode configuration used in this work is shown schematically in Fig. 1. The glassy carbon electrode (GCE) was modified successively with MWCNT dispersed

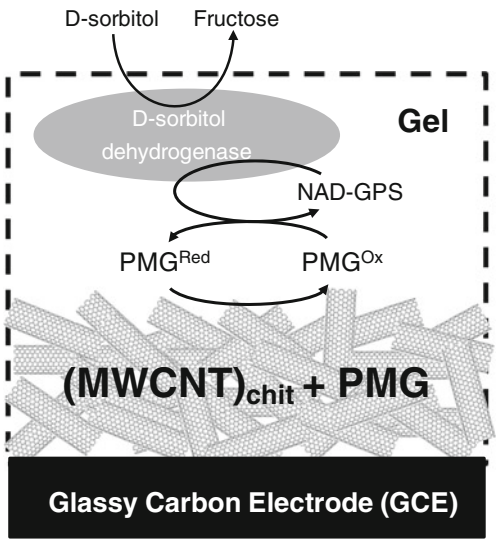

Fig. 1 Schematic illustration of the electrode configuration used in this study. Electrochemical oxidation of NADH was catalyzed on the carbon nanotube surface modified with electropolymerized poly(methylene green) ($(MWCNT)_{chit}$ + PMG) and NAD^+ was covalently linked with NAD-GPS to the sol–gel used for encapsulation of D-sorbitol dehydrogenases (DSDH)

in chitosan, $(MWCNT)_{chit}$, poly(methylene green), PMG, and, finally, with a sol–gel thin film containing both cofactor (NAD-GPS) and D-sorbitol dehydrogenase (DSDH).

Preparation of GCE/$(MWCNT)_{chit}$ + PMG

Glassy carbon electrodes (GCE) were first polished on wet emery paper (4,000), then with Al_2O_3 powder (0.05 μm, Buehler). Electrodes were then washed with ethanol and water. Chitosan-MWCNT suspension was prepared by dispersing 1.0 mg MWCNT into 1 mL of chitosan solution (0.2 % in 0.05 molL^{-1} acetic acid solution). An aliquot (5 μL) of $(MWCNT)_{chit}$ suspension was then deposited on to the GCE surface and left to dry at room temperature to furnish GCE/$(MWCNT)_{chit}$.

Electropolymerization of MG on GCE/$(MWCNT)_{chit}$ or bare GCE was achieved by cycling the potential of the electrode from −0.5 to 1.2 V at a scan rate of 50 mVs^{-1} in 0.1 molL^{-1} pH7.0 PBS containing 0.5 mmolL^{-1} MG and 0.1 molL^{-1} KCl. Deposition was stopped after 10 cycles and electrodes were thoroughly rinsed with high-purity water and dried at room temperature before use.

Preparation of GCE/$(MWCNT)_{chit}$ + PMG + $(DSDH)_{gel}$

A typical silica sol was prepared by dissolving 2.125 g TEOS in 2 mL water and 2.5 mL 0.01 molL^{-1} aqueous HCl, which were continuously stirred for 12 h and then diluted six times with water for further use. This sol (20 μL) was mixed with 10 μL PEI solution (20 % (*w*/*w*), pH9.0), 10 μL water, and 20 μL DSDH (10 mgmL^{-1}). An aliquot (5 μL) of this resulting sol was drop-coated on to GCE/$(MWCNT)_{chit}$ + PMG. The solution was then left to dry at 4 °C overnight.

Preparation of GCE/$(MWCNT)_{chit}$ + PMG + $(DSDH/NAD\text{-}GPS)_{gel}$

NAD–GPS solution was typically prepared by mixing 25 mg NAD^+ and 37.5 mg GPS in 400 μL Tris–HCl buffer solution (pH7.5) at room temperature under stirring for 14 h. 20 μL of above mentioned sol was mixed with 10 μL PEI solution (20 % (*w*/*w*), pH9.0), 10 μL NAD-GPS solution, and 20 μL DSDH (10 mgmL^{-1}). An aliquot (5 μL) of the resulting sol was deposited on to GCE/$(MWCNT)_{chit}$+PMG. The solution was then left to dry at 4 °C overnight. A control experiment was also performed by simply encapsulating the NAD^+ cofactor. This last configuration was called GCE/$(MWCNT)_{chit}$ + PMG + $(DSDH/NAD^+)_{gel}$.

All prepared electrodes were rinsed thoroughly with water and stored in Tris–HCl buffer solution for 15 min before electrochemical measurements.

Results and discussion

Electrocatalytic oxidation of NADH at GCE/$(MWCNT)_{chit}$ + PMG

Electroanalytical applications of NAD-dependent dehydrogenase are based on electrochemical determination of the reduced cofactor (NADH) which is produced during the enzymatic reaction with the analyte to be determined. Development of effective systems for oxidation of NADH to enzymatically active NAD^+ is a critical step in investigation of application of the dehydrogenase in reagentless biosensors [1]. Electropolymerization of methylene green as electrocatalyst for NADH oxidation was originally reported for modification of flat electrodes [36]. Recently, this approach was also extended to the functionalization of carbon nanotubes [40, 41].

Here we first deposited a layer of MWCNT dispersed in chitosan before electropolymerization of methylene green. Figure 2 shows a typical electropolymerization process for MG on GCE/$(MWCNT)_{chit}$; it consists of ten potential cycles from −0.5 to +1.2 V. The peak current observed between −0.3 and +0.3 V increased with the number of cycles, which indicates that continuous electrochemical polymerization enables immobilization of increasing amounts of electroactive methylene green species.

Fig. 3a (curve 1) shows a typical cyclic voltammogram obtained with GCE/$(MWCNT)_{chit}$ + PMG in Tris–HCl solution at pH9. This response was rather complex and, in agreement with observations reported in the literature [38, 41], several successive redox peaks were observed between −0.4 and +0.1 V. Addition of NADH led to modification of the cyclic voltammogram with the oxidation current increasing at potentials higher than 0 V (Fig. 3a, curve 2). Figure 3b shows a comparative amperometric response recorded at +0.2 V, on

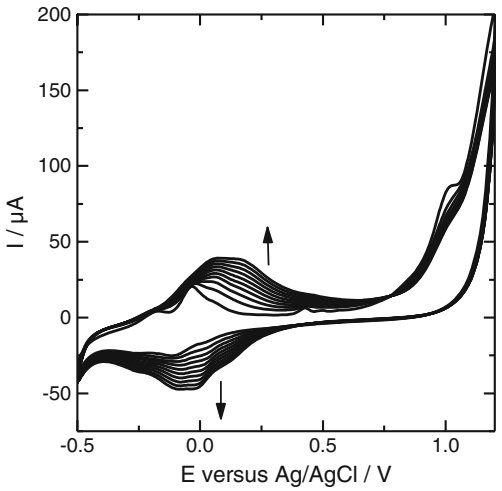

Fig. 2 Electropolymerization of methylene green on the GCE/$(MWCNT)_{chit}$ obtained by cycling between −0.5 and 1.2 V at a scan rate of 50 mVs^{-1} in 0.1 molL^{-1} pH7.0 PBS containing 0.5 mmolL^{-1} MG and 0.1 molL^{-1} KCl

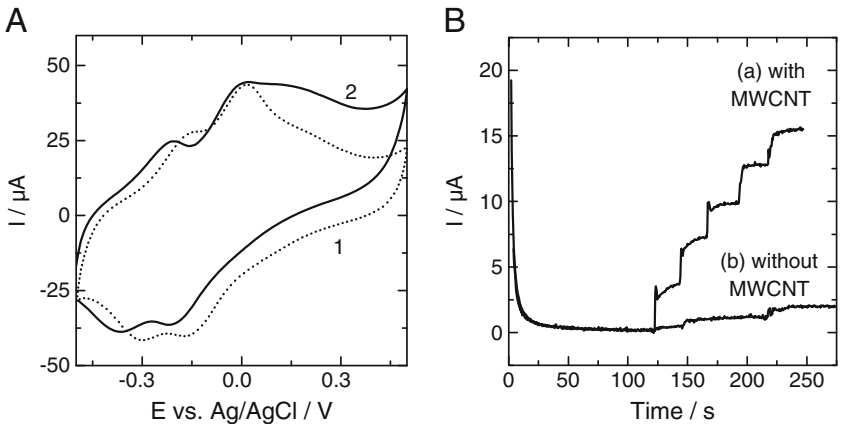

Fig. 3 (**a**) Cyclic voltammograms recorded at a scan rate of 50 mVs^{-1} at GCE/$(MWCNT)_{chit}$+PMG (*1*) before and (*2*) after addition of 1.2 $mmolL^{-1}$ NADH. (**b**) Amperometric responses obtained at an applied potential of +0.2 V vs Ag/AgCl with (*a*) GCE/$(MWCNT)_{chit}$+PMG and (*b*) GCE/PMG on successive addition of 0.2 $mmolL^{-1}$ NADH. All measurements were performed in 0.1 $molL^{-1}$ Tris–HCl buffer (pH9)

successive additions of NADH (0.2 $mmolL^{-1}$), for glassy carbon electrodes modified with PMG in the presence (curve a) or absence of MWCNT (curve b). The catalytic response was observed in both cases, however this response was almost one order of magnitude higher when poly(methylene green) was deposited on the MWCNT. This can be explained by the high rate of electron transfer enabled by the large electroactive surface area of carbon nanotubes. Amperometric detection was also possible at an applied potential of 0 V (vs Ag/AgCl), but sensitivity was not as good as at +0.2 V (vs Ag/AgCl). The presence of the carbon nanotubes also substantially improved the stability of the electrochemical response for mediated oxidation of NADH, as reported elsewhere [33]. These results confirm the great interest in using CNT to improve the electrocatalytic performance of PMG in regeneration in NAD^+.

Sol–gel deposition on the surface of GCE/$(MWCNT)_{chit}$ + PMG

The excellent sensitivity of GCE/$(MWCNT)_{chit}$ + PMG in the detection of NADH at low overpotential makes this combination attractive for development of dehydrogenase-based amperometric biosensors. The GCE/$(MWCNT)_{chit}$ + PMG system was first evaluated in combination with D-sorbitol dehydrogenase (DSDH) immobilized in a sol–gel thin film deposited on the surface of functionalized carbon nanotubes. To determine the feasibility of this method of detection, a first series of experiments was performed with the NAD^+ cofactor in solution. Typical cyclic voltammetric responses obtained from this modified electrode are shown in Fig. 4a, in the absence (solid line) and presence of 2, 4, or 6 $mmolL^{-1}$ D-sorbitol (dashed and dotted lines). In the absence of D-sorbitol, only the electrochemical signal of MG was observed. Addition of 2 $mmolL^{-1}$ D-sorbitol induces the oxidation peak current increase at +0.2 V, and this trend continues with increasing D-sorbitol concentration. Figure 4b shows the amperometric response of different films to D-sorbitol in the same Tris–HCl buffer solution containing 1 $mmolL^{-1}$ NAD^+ at an applied potential of +0.2 V. The background current was left to decay to a steady value before the aliquots of D-sorbitol solution were added. Obvious current responses were observed on the GCE/$(MWCNT)_{chit}$ + PMG + $(DSDH)_{gel}$, and this signal

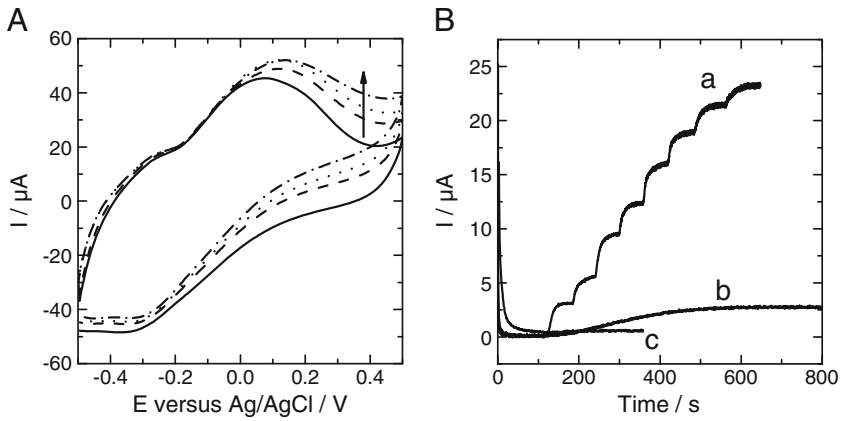

Fig. 4 (**a**) Cyclic voltammograms obtained at a scan rate of 50 mVs^{-1} for GCE/$(MWCNT)_{chit}$+PMG+$(DSDH)_{gel}$ in the absence (*solid line*) and presence of 2, 4, or 6 $mmolL^{-1}$ D-sorbitol (*dashed and dotted lines*). (**b**) Amperometric responses obtained with (*a*) GCE/$(MWCNT)_{chit}$+PMG+$(DSDH)_{gel}$; (*b*) GCE/PMG+$(DSDH)_{gel}$; (*c*) GCE/$(MWCNT)_{chit}$+$(DSDH)_{gel}$ on successive addition of D-sorbitol (0.5, 0.5, 1, 1, 2, 2, 3, and 3 $mmolL^{-1}$). All measurements were performed in 0.1 $molL^{-1}$ Tris–HCl buffer (pH9) containing 1 mmol L^{-1} NAD^+ at an applied potential of +0.2 V vs Ag/AgCl

increased with each addition of D-sorbitol (curve a). This corresponds to electrochemical oxidation of the NADH cofactor produced by the encapsulated DSDH while oxidizing D-sorbitol. Curves b and c show the responses obtained by use of films prepared in the absence of MWCNT (but with PMG on glassy carbon, GCE + PMG + $(DSDH)_{gel}$) and in the absence of PMG (but in the presence of MWCNT—$GCE/(MWCNT)_{chit}$ + $(DSDH)_{gel}$). As expected, no change in the electrochemical signal was observed when D-sorbitol was added in the absence of PMG (curve c), because of the lack of electrocatalytic oxidation of NADH. Only a small increase in the electrochemical signals was observed for $GCE+PMG+(DSDH)_{gel}$ (curve b) after addition of D-sorbitol to the solution. So, only the system including both MWCNT and PMG had satisfactory sensitivity to D-sorbitol. This result can be ascribed to the synergetic effects of both increased electroactive surface area, because of the use of MWCNT for PMG deposition and the catalytic properties of the PMG mediator. This electrocatalytic system was not limited by the presence of the sol–gel layer.

To further characterize the electrodes prepared in this study, SEM and AFM images were obtained for $GCE/(MWCNT)_{chit}$, $GCE/(MWCNT)_{chit}$ + PMG, and $GCE/(MWCNT)_{chit}+PMG+(DSDH)_{gel}$ (Fig. 5). Carbon nanotubes were well defined; their diameter was easily resolved by SEM and AFM. Deposition of poly(methylene green) did not alter the quality of the film, which could be indicative of homogeneous deposition of PMG on the carbon nanotube assembly. EDS analysis showed that no sulfur was present in $GCE/(MWCNT)_{chit}$, i.e., before PMG deposition, whereas a small amount of this element (0.8 % *w*/*w*) could be detected after PMG deposition, because it is present in the methylene green monomer. Finally, deposition of the sol–gel layer containing DSDH led to complete coverage of the carbon nanotubes, as proved by both AFM and SEM.

All the electrochemical experiments discussed in this section were conducted with the NAD^+ cofactor in the solution, and thus freely diffusing in the sol–gel matrix. The next section will discuss possible immobilization of NAD^+ in the sol–gel layer to produce a reagentless sensor.

Production of a reagentless device

Fabrication of a dehydrogenase-based reagentless electrochemical biosensor requires that both the enzyme and its cofactor are co-immobilized on the electrode surface. A strategy recently developed by our group involves reaction of the adenine group of NAD^+ with the epoxy group of glycidoxypropylsilane (GPS) [13, 14] leading to NAD–GPS. This molecule is produced in situ during the first stage of the sol–gel process. the starting sol, containing both silane precursors (TEOS and GPS) and NAD^+ was then used for dehydrogenase encapsulation.

Here, $GCE/(MWCNT)_{chit}$ + PMG was coated with such a sol–gel layer, enabling co-immobilization of DSDH and NAD^+ ($(DSDH/NAD–GPS)_{gel}$). Figure 6a shows the electrochemical response of this modified electrode before

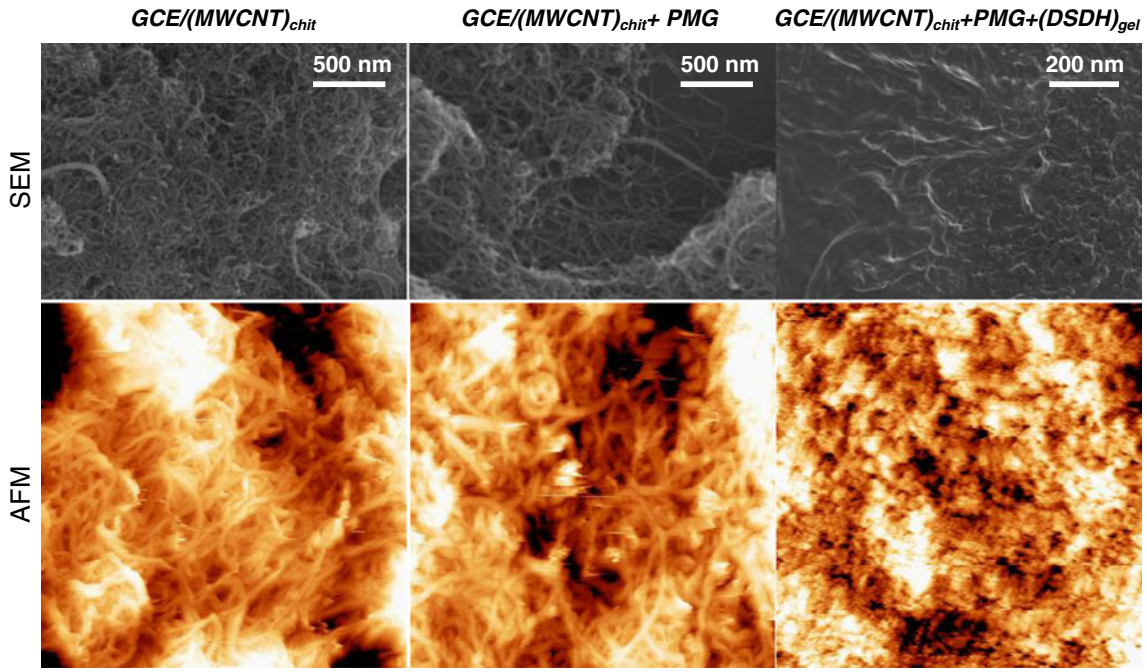

Fig. 5 SEM and AFM characterization of $GCE(MWCNT)_{chit}$, $GCE(MWCNT)_{chit}$+PMG, and $GCE(MWCNT)_{chit}$+PMG+$(DSDH)_{gel}$. The size of all AFM images is 2 μm×2 μm

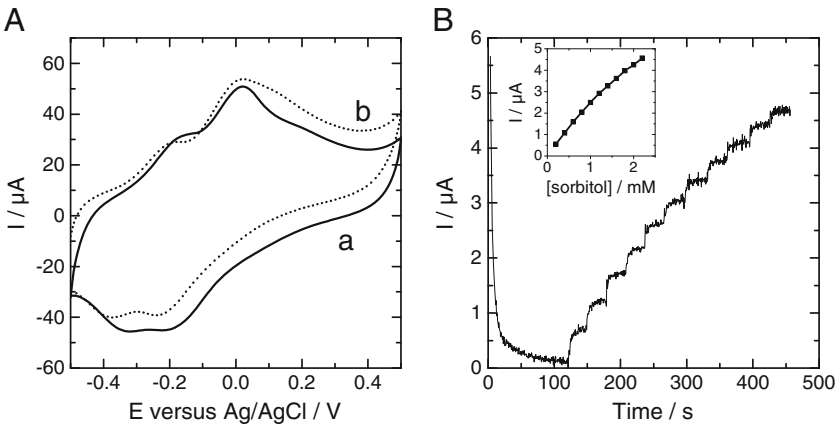

Fig. 6 (**a**) Cyclic voltammograms recorded with GCE/$(MWCNT)_{chit}$+ PMG+$(DSDH/NAD\text{-}GPS)_{gel}$, (*a*) in the absence and (*b*) in the presence of 2.2 mmolL^{-1} D-sorbitol. Potential scan rate 50 mVs^{-1}. (**b**) Amperometric responses recorded at an applied potential of+0.2 V on successive addition of 0.2 mmolL^{-1} D-sorbitol to a stirred solution. The *inset* shows the corresponding calibration plot

(curve a) and after (curve b) addition of D-sorbitol to the solution. In the absence of D-sorbitol, only the electrochemical complex signal of PMG on MWCNT was observed. Addition of D-sorbitol induced an increase of the anodic current and a decrease of the cathodic current, as expected for electrocatalytic processes. For this electrode configuration, electrocatalysis was related to electrochemical oxidation by the PMG mediator of the immobilized NADH cofactor produced by the encapsulated DSDH while oxidizing D-sorbitol.

Figure 6b shows amperometric responses of the modified electrode to D-sorbitol in a stirred solution. An increasing electrocatalytic response was obtained upon successive addition of 0.2 mmolL^{-1} D-sorbitol. The electrode response varied linearly with the concentration of D-sorbitol from 0.2 to 1.2 mmolL^{-1} and then started to level off at higher concentrations (inset in Fig. 6b). These results indicate that the cofactor immobilized in the gel was sufficiently mobile to reach the catalytic site of dehydrogenase, to perform oxidation of D-sorbitol, and was also able to interact with $GCE/(MWCNT)_{chit}$ + PMG to enable regeneration.

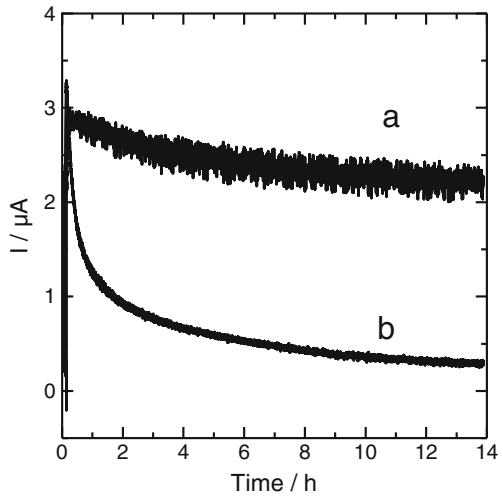

Fig. 7 Chronoamperometric responses recorded at an applied potential of +0.2 V (vs Ag/AgCl) with (*a*) $GCE/(MWCNT)_{chit}$+PMG+$(DSDH/NAD\text{-}GPS)_{gel}$ and (*b*) $GCE/(MWCNT)_{chit}$+PMG+$(DSDH/NAD^{+})_{gel}$. Measurements were performed in 0.1 molL^{-1} Tris–HCl buffer (pH9) containing 1 mmolL^{-1} D-sorbitol for 14 h under convective conditions

In view of potential analytical applications it was necessary to characterize this bioelectrode with regard to linear range, sensitivity, and detection limits. These characteristics were determined by recording the amperometric response at an applied potential of +0.2 V, as a function of D-sorbitol concentration. The calibration plot for D-sorbitol oxidation was linear for D-sorbitol concentrations between 0.2 and 1.2 mmolL^{-1}, with sensitivity of 8.7 μA$mmol^{-1}Lcm^{-2}$. The detection limit for D-sorbitol was estimated to be 0.11 mmolL^{-1} on the basis of a signal-to-noise ratio of 3. The sensitivity of the bioelectrode developed here was similar to or better than that previously reported for D-sorbitol dehydrogenase-based biosensors [16, 43–46]. The detection limit observed with our bielectrode is even lower than that obtained by Cambell et al. with a honeycomb-like structure of active carbon [47]. Comparison with our recently published work on sol–gel-based reagentless sensors, indicates that sensitivity was of the same order of magnitude, but the linear range was smaller in the absence of diaphorase (this work) than in the presence of this redox protein [13, 14].

The operational long-term stability of modified electrodes on continuous use in hydrodynamic conditions was also evaluated. Response to 1 mmolL^{-1} D-sorbitol of bioelectrodes obtained by use of simply encapsulated

Table 1 Recovery data for D-sorbitol determination. Candy solution prepared by dissolving 0.1 g D-sorbitol-free candy in 5 mL Tris–HCl buffer solution was spiked with different amounts of D-sorbitol

D-Sorbitol concentration		Recovery (%)	R.S.D. (%) (n=3)
Added ($\times10^{-3}$ molL^{-1})	Determined ($\times10^{-3}$ molL^{-1})		
0.20	0.212	105	3.8
0.60	0.59	98	9.3
1.20	1.12	93	5.4

R.S.D. is relative standard deviation

NAD^+ (without GPS in the starting sol, curve b) and with NAD^+ chemically linked to the film (NAD–GPS, i.e., with GPS in the starting sol, curve a) is compared in Fig. 7. For both electrodes the response was comparable (approx. 3 μA) at the beginning of the experiment; however, the electrode with the immobilized cofactor had much better operational stability, i.e., more than 75 % of current response was retained after continuous 14-h-long experiments in a stirred solution (curve a). In contrast , the electrode prepared by simple encapsulation of NAD^+ had very low stability (curve b)—the electrocatalytic response was lost during the first 6 h of experiments. This can be explained by rapid leaching of NAD^+ from the sol–gel layer to the solution.

In summary, the immobilized cofactor in the sol–gel matrix can be safely regenerated by PMG deposited on MWCNT, and the stability of the electrochemical response in continuous use was found to be comparable with that in previous work involving diaphorase for electroenzymatic regeneration of NAD^+ immobilized in the sol–gel [13, 14] or with that for electrocatalytic regeneration of NAD^+ covalently linked to chitosan [11, 12].

As a preliminary study of the validity of the proposed electrochemical biosensor, recovery of D-sorbitol in a complex matrix was evaluated. For this purpose D-sorbitol-free candy was chosen as matrix and spiked with different amounts of D-sorbitol before analysis (Table 1). The results indicate that the suggested D-sorbitol biosensor might be useful for determination of D-sorbitol because recovery was satisfactory at 93–105 %.

The sol–gel carbon nanotubes–poly(methylene green) matrix developed in this work could easily be modified for development of reagentless biosensors based on other dehydrogenases also. For example, the same biosensor configuration using glucose dehydrogenase (from *Pseudomonas* sp.) had linear response from 0.6 to 2.6 $mmol L^{-1}$ with sensitivity of 6.4 $\mu A mmol^{-1} L cm^{-2}$ (data not shown).

Conclusions

A reagentless D-sorbitol biosensor based on D-sorbitol dehydrogenase immobilized in a sol–gel carbon nanotubes–poly(methylene green) composite has been developed. In this system, the sol–gel matrix was used for co-immobilization of D-sorbitol dehydrogenase and NAD^+ cofactor on the surface of carbon nanotubes functionalized with poly(methylene green). This device enables selective determination of D-sorbitol at 0.2 V with 8.7 $\mu A mmol^{-1} L cm^{-2}$ sensitivity, 0.11 $mmol L^{-1}$ detection limit, and good stability with stirring in continuous use for more than 14 h. The reagentless sensor was successfully applied to the determination of D-sorbitol in candy. The same immobilization strategy could be extended to other dehydrogenases.

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