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Co-Immobilization of Dehydrogenases and Their Cofactors in Electrochemical Biosensors

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Enzyme-based reagentless biosensors were developed using the model system of glucose dehydrogenase (GDH) and its nicotinamide adenine dinucleotide cofactor (NAD^+). The biosensors were prepared following an approach similar to the concept of molecular imprinting. To this end, the N^1-carboxymethyl-NAD^+ species were covalently attached to poly-amino-saccharide chains of chitosan (CHIT) and allowed to interact with GDH in an aqueous solution. The bioaffinity interactions between the NAD^+ and GDH were secured by crosslinking the system with the glutaric dialdehyde (GDI)-modified CHIT. Electron conductive films of such CHIT-NAD^+-GDH-GDI-CHIT macro-complexes (MC) were prepared on glassy carbon (GC) electrodes by adding carbon nanotubes (CNT) and evaporating water. Electrochemical analysis of the GC/CNT-MC electrodes revealed that, in contrast to the oxidase-based electrodes, they acted as oxygen-independent reagentless biosensors. The application of Nafion to such biosensors predictably improved their selectivity and, unexpectedly, enhanced their sensitivity by an order of magnitude.

The focus of the present paper is on dehydrogenase-based reagentless biosensors, i.e. biosensors that can operate in samples containing no extra reagents (e.g. enzymes, cofactors, mediators). In particular, the synthetic protocols developed in the present work are suitable for the construction of electrochemical biosensors based on a large group of the NAD-dependent dehydrogenase enzymes. For the sake of brevity, NAD-dependent dehydrogenase enzymes will be referred to as dehydrogenase enzymes throughout the paper. The advantage of dehydrogenase enzymes over the oxidases is that they are oxygen independent, more abundant, and more substrate specific. These features translate, in principle, into the biosensors that are free of oxygen interferences, allow for a wide range of possible substrates (analytes) to be measured, and provide a higher level of molecular recognition. Thus, a successful use of dehydrogenases is expected to open up new fields of applications both in analysis and bioelectronics.

However, dehydrogenase-based biosensors are not yet a success story. This can be attributed to some restrictions in the use of dehydrogenases for the development of reagentless electrochemical biosensors. The primary limitation is that, in contrast to oxidases, which have redox cofactors tightly bound within their molecules, the cofactors are not bound to dehydrogenase molecules. Therefore, unlike the case of oxidase-based biosensors, dehydrogenase-based systems cannot be readily incorporated into reagentless devices. The latter systems require that both a dehydrogenase and its cofactor are immobilized in such a

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way that a cofactor has easy access to an enzyme. Another limitation in using of dehydrogenases is the fact that their cofactors are recycled only at high potentials at most electrodes, which leads to interferences from redox active species other than the enzyme substrate. Consequently, and predictably, the development of dehydrogenase-based reagentless biosensors has been rather slow. Typically, such biosensors have been designed using redox mediators to recycle enzyme cofactors and immobilizing the dehydrogenases and their cofactors by entrapping them in carbon pastes,[1–8] membranes,[9–15] composite materials,[16–18] macroporous electrodes,[19] and assembled layers.[20,21]

The present paper describes the immobilization of glucose dehydrogenase (GDH) and its cofactor NAD^+ on the scaffolds of a biopolymer chitosan (CHIT). The novelty of this approach is that the dehydrogenase and the cofactor are covalently attached to polymeric chains on the surface of electrode and still have enough freedom of movement to react with each other and provide a sustainable amount of current in the presence of glucose in a solution.

EXPERIMENTAL SECTION

Reagents

Chitosan (CHIT, MW ~ 1 × 106 Da; ~80 % deacetylation), nicotinamide adenine dinucleotide (NAD^+, cat.# N0632, λ = 259 nm), iodoacetic acid, NADH, glucose dehydrogenase (GDH, from pseudomonas sp., EC 1.1.1.47, 275 units mg^{-1}), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), Nafion (5 wt. %), methanol, ethanol, ethyl ether and glutaric dialdehyde (GDI, 25 wt. % solution in H_2O) were purchased from Sigma-Aldrich. Multi-walled carbon nanotubes (CNT, ~95 % nominal purity) were purchased from Nanolab (Brighton, MA). The dialysis tubings (1.2–1.4 × 10^4 MWCO), NaH_2PO_4•H_2O, Na_2HPO_4, HCl, and NaOH were from Fisher. All solutions were prepared using 18-MΩ-cm deionized water that was purified with a Barnstead NANOpure cartridge system.

Electrochemical Measurements

A CHI 832B workstation (CH Instruments, Inc.) was used to collect electrochemical data. In order to increase the ratio of signal to noise, all of the experiments were done in a Faraday cage (BAS C-2 cell stand). The measurements were performed in a three-electrode system. A 3.0-mm-diameter glassy carbon (GC) disk served as the working electrode (Bioanalytical Systems, Inc.; BAS), a platinum wire was used as the auxiliary electrode, and the reference electrode was the Ag/AgCl/3MNaCl (BAS). The glassy carbon electrodes were wet polished on an Alpha A polishing cloth (Mark V Lab) with successively smaller particles (0.3 and 0.05 μm diameter) of alumina. The alumina slurry was removed from the electrode surface by a 30-s ultrasonication in deionized water and methanol.

All experiments were performed at room temperature (20 ± 1 °C) using the pH 7.40 phosphate buffer solution (0.10 M) as a background electrolyte. The experiments were repeated minimum three times and the means of measurements are presented with the relative standard deviations (RSD).

Preparation of CHIT, CHIT-GDI, and CHIT/CNT Solutions

The solutions were prepared following our recent protocols. Briefly, the CHIT solutions were prepared by dissolving chitosan flakes in hot 0.05 M HCl and filtering with a syringe filter.[22] The CHIT-GDI solutions, used for the covalent immobilization of GDH, were prepared by reacting chitosan solution with the glutaric dialdehyde and purifying the CHIT-GDI product using dialysis.[23] In order to react only one aldehyde group of GDI, a high

molar ratio of GDI to chitosan glucosamine units (200:1) was used.[24] The colloidal suspensions of CNT in CHIT solutions were prepared by sonication.[25]

Synthesis of N^1-carboxymethyl-NAD^+

N^1-carboxymethyl-NAD^+ was synthesized following the modified literature method.[26,27] Our modification eliminated two synthetic steps by judicious selection of the pH conditions. Solutions of 2 g of NAD^+ in 15 mL water and 4 g of iodoacetic acid in 20 mL water were mixed together and the pH adjusted to 6.5 using 3.0 M NaOH. During the first few hours, the pH was continuously monitored and adjusted intermittently as necessary. Subsequently, the reaction mixture was kept in dark at room temperature for 20 days. During that time, the solution, which was initially colorless, turned gradually pink and, finally, orange-red. At this point, the solution was acidified to pH 3.0 using 3.0 M HCl, poured into a 10-fold excess of chilled ethanol, and kept at − 20 °C overnight to form a precipitate. The precipitate was filtered, washed three times with ethanol and ethyl ether, and dried under vacuum to produce a pink-red powder.

Synthesis of CHIT-NAD^+

The solution of 0.20 g N^1-carboxymethyl-NAD^+ in 10.0 mL water was mixed with 10.0 mL of 0.20 wt. % CHIT solution, and 0.20 g of EDC catalyst was slowly added. The pH was adjusted to 4.70 with 0.50 M HCl and continuously monitored and intermittently adjusted during the first few hours. After 12 h at room temperature, the solution was dialysed against four 1-L batches of HCl solution (pH 4.5) for 12 h each in order to remove the unreacted NAD^+ derivative. The progress of species removal was monitored by using UV-visible spectrophotometry to follow a decrease in the NAD^+ adenine absorption band. At the end of the dialysis, the volume of the dialyzed mixture slightly increased from initial 20.0 to 21.3 mL, which changed the concentration of CHIT from initial 0.10 to 0.094 wt. %.

Spectroscopic Measurements

The mass spectra of the N^1-carboxymethyl-NAD^+ and CHIT-NAD^+ solutions were recorded with a Finnigan LCQ Duo ion-trap mass spectrometer utilizing electrospray ionization in positive ion detection mode. The mass range recorded was from 150 to 2000 m/z. Samples were directly infused at a rate of 50 μL min^{-1}. A spray voltage of 3.5 kV was applied to a solution of the product in a water-methanol mixture (50:50 vol. %) that contained 0.10 vol. % acetic acid. Collision induced dissociation (CID) experiments were performed using an isolation width of 3.0 m/z. The collision energy was varied in order to produce a parent ion signal smaller than 10 % of the total ion current. The source CID at energy of 25.0 V was also performed on the CHIT-NAD^+ solutions. Serial CID experiments were performed until the daughter ion intensities dropped bellow 3:1 S/N.

Electronic spectra were recorded with a HP-8453 UV-visible diode array spectrophotometer using a quartz cuvette with a path length of 1.0 cm.

Preparation of Reagentless Biosensors

The procedure involved a sequence of solution mixing steps on the surface of a glassy carbon electrode. After extensive optimization studies, the best sensitivity of the biosensors toward glucose was achieved with the following solutions: (A) 30.0 μL of CHIT-NAD^+ solution (0.094 wt. % CHIT, 1.5 mM immobilized NAD^+), (B) 5.0 μL of GDH solution (12 mg mL^{-1}), (C) 20.0 μL of 0.10 wt. % CHIT-GDI solution, and (D) 10 μL of CNT suspension (1.0 mg mL^{-1}) in 0.10 wt. % CHIT solution.

First, the mixture of solutions A and B was allowed to equilibrate for 30 min in order to establish the CHIT-NAD^+-GDH interactions. A 20-μL aliquot of water was added, if

necessary, to prevent the drying. Second, solution C was admixed in order to form the CHIT-NAD^+-GDH-GDI-CHIT macro-complexes (MC). Again, the mixture was kept liquid and equilibrated for 30 min. Finally, suspension D was added in order to adsorb the MC on CNT. After evaporation of water at room temperature for 3 h, the CNT-MC film electrodes were soaked in a stirred phosphate buffer solution (pH 7.40) for 1 h in order to remove weakly attached components. After rinsing with water, the wet electrodes were coated with 20 μL of 5.0 wt. % Nafion solution in order to improve their selectivity. The electrodes were finally dried at room temperature for 1 h. Such GC/CNT-MC/Nafion film electrodes (biosensors) displayed the best combination of high substrate sensitivity, short response time, least interferences, and good stability. All of the biosensors were soaked in the background electrolyte solution for 1 h before their first use. When not in use, they were stored in a freezer (−20°C).

RESULTS AND DISCUSSION

Covalent Attachment of NAD^+ to CHIT Chains

The CHIT-NAD^+ species (Figure 1) were synthesized by reacting the carboxylic groups of N^1-carboxymethyl-NAD^+ with amino groups of CHIT in the presence of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride catalyst. The CHIT- NAD^+ product was dialyzed, in order to remove the catalyst species and any unbound NAD^+, and characterized by the mass spectrometry, UV-visible spectrophotometry, and amperometry.

Figure 2 shows a collision induced dissociation (CID) mass spectrum of the CHIT-NAD^+ product. The ions at m/z equal to 564.1, 582.1, 704.1, and 722.1 correspond to fragments of NAD^+ (m/z = 722.1) that dissociated from the CHIT-NAD^+ species. The explicit structures of these, and other, fragments are provided in the Supplemental Information section. Considering that the unbound NAD^+ was removed by dialysis, the detection of NAD^+ in the sample was ascribed to covalent linkage to CHIT chains. This was further corroborated by the fact that the NAD^+ was not detected in the absence of the source CID potential.

The amount of NAD^+ immobilized on CHIT chains was determined from the absorbance of the dialyzed CHIT-NAD^+ solution at 259 nm. For the 40 times diluted solution, the absorbance A_{259} was equal to 0.588 ± 0.014 AU. According to the NAD^+ calibration plot (A_{259} = 15.98 C_{NAD+} (mM) – 0.008, R^2 = 0.999) an absorbance of 0.588 corresponded to ~1.5 mM of NAD^+ in the undiluted CHIT- NAD^+ solution. The concentration of chitosan glucosamine units in the undiluted solution was equal to ~4.7 mM, based on the concentration of chitosan (0.094 wt. %), percent of chitosan deacetylation (~80 %), density of solution (1.0 g mL^{-1}), and the molar mass of glucosamine units (161 g mol^{-1}). This indicated that, on average, the molar ratio of NAD^+ to glucosamine units in the CHIT-NAD^+ species was approximately 1:3.

Figure 3 shows the response of a GC/CHIT-CNT electrode (E = 0.40 V) to an addition of glucose to a solution that contained the CHIT-NAD^+ species and enzyme GDH. The addition of glucose to the solution triggered the flow of faradaic current through the electrode. Since glucose was not directly oxidized at the electrode, the current was ascribed to the electrooxidation of NADH at CNT.[25] The NADH was generated by the enzymatic oxidation of glucose according to the reaction

$$\mathrm{Glucose + CHIT{-}NAD^+ \xrightarrow{GDH} Gluconolactone + CHIT{-}NADH} \quad (1)$$

The gradual increase in current in Figure 3, after the fast initial rise in current, may be ascribed to the gradually improved access of the polymeric CHIT-NADH species to the

CNT due to the swelling of the CHIT-CNT gel film on the surface of the electrode. The presence of the current indicated that the chemical modification of NAD^+ and its covalent attachment to CHIT chains did not deactivate the cofactor. In addition, it demonstrated that the bioaffinity of GDH for N^1-linked immobilized cofactor was sufficient to carry out the enzymatic reaction 1.

Characterization of Reagentless Biosensors

Figure 4 shows a typical hydrodynamic voltammogram recorded at the GC/CNT-MC/Nafion biosensors. It illustrates that they yield a maximum response to glucose at potentials ≥ 0.40 V. More importantly, it reveals that the enzyme and its cofactor communicate with each other despite the expected steric hindrances due to their covalent immobilization on the electrode surface. The significance of the CNT in the biosensors is two-fold. First, they lower the overpotential for NADH oxidation by ~300 mV, when compared to the bare glassy carbon. Second, the electrooxidation of NADH takes place at a three-dimensional network of CNT, which facilitates the charge propagation through the surface film and, thus, increases the sensitivity toward the NADH.

The mode of Nafion casting influenced the sensitivity of biosensors to glucose. This is illustrated in Figure 5, where the response of uncoated GC/CNT-MC electrode (curve a) is provided for comparison purposes. Casting the Nafion on dry GC/CNT-MC electrodes practically eliminated the glucose signal (curve b). Apparently, the Nafion film precluded the swelling of a CNT-MC film in such electrodes (‘dry’ electrodes) after subsequent exposure to an aqueous solution. The swelling of chitosan-based films is a necessary condition for the fast ion transport in order to support a flow of discernible amount of current.[22]

The response to glucose was spectacularly enhanced (Figure 5, curve c) when the Nafion was cast on wet GC/CNT-MC electrodes that were soaked for 1 h in the background electrolyte solution before the application of Nafion. For example, such Nafion coated electrodes (‘wet’ electrodes) yielded a current of 330 nA (RSD = 27 %, N = 26) at 0.40 V in a solution of 5.0 mM glucose. Under the same conditions, the electrodes without the Nafion (GC/CNT-CM) yielded much smaller current of 31 nA (RSD =21 %, N = 26). The role of Nafion in the current enhancement is not clear at present. The preconcentration of glucose in the CNT-MC/Nafion film is rather unlikely because the hydrophilic glucose molecules are neutral under the experimental conditions used. Indeed, the Nafion film partially rejects such molecules as indicated by a decreased current of a neutral acetaminophen (Figure 6b). Nafion films are known to preconcentrate positively charged species that display a degree of hydrophobicity. The improved transport in the CNT-MC/Nafion film is also doubtful as a source of the current increase because the response time to glucose ($t_{90\%}$) increased ~6 times, from 16 to 92 s on average, when Nafion was applied to the GC/CNT-MC electrodes. However, the increase in the response time may be in part due to a thicker surface film with the Nafion overlayer. Perhaps, the increase in the current is a result of a synergy between the chitosan and Nafion that results in facilitated access of NADH to CNT. Both, chitosan[25] and Nafion[28] are known to facilitate the dispersion of CNT. However, more studies are needed in order to determine the mechanism of current enhancement in such a system.

The linear and non-linear calibration plots were analyzed. The current flowing through the GC/CNT-MC/Nafion biosensors was a linear function of glucose concentration in the range from 0.02 to 2.0 mM (R^2=0.994). The slope of the current vs. concentration (sensitivity) was equal to 1.8 mA M^{-1} cm^{-2} assuming a geometric surface area of 3-mm diameter disk electrode

In the wider concentration range 0.020–20.0 mM, the biosensor current was a non-linear function of glucose concentration (Figure 5, inset). The non-linear calibration plot (solid line 1) was obtained by fitting of 15-point experimental sets of current (I)-concentration (C) data to the exponential equation ($R^2 = 0.999$)

$$I = I_0 + A_1[1 - \exp(-C/a_1)] + A_2[1 - \exp(-C/a_2)] \quad (2)$$

where $I_0 = 0.00126 \pm 0.00079$, $A_1 = 0.45 \pm 0.09$, $a_1 = 8.2 \pm 1.5$, $A_2 = 0.32 \pm 0.10$, and $a_2 = 2.9 \pm 0.4$. This illustrated that, with the existing computing technology, the extended data sets can be easily used in the quantification of enzyme substrate.

Figure 6 (line A) shows that, as anticipated, Nafion practically eliminated the interferences from anionic redox active species (ascorbate, urate) and largely diminished the interference from neutral molecules (acetaminophen). Without the Nafion, the GC/CNT-MC electrodes displayed large currents due to the oxidation of interfering species (line B). The inset in Figure 6 demonstrates a major advantage of a dehydrogenase-based biosensor, which is the lack of interferences from oxygen.

The GC/CNT-MC/Nafion biosensors displayed a good operational stability during continuous 25-h long experiments (Figure 7). The shelf-life stability studies showed that the biosensors maintained a constant glucose signal (±10 %) during first 4–5 days and retained ~60 % of their response to glucose after 48 days of storage in air at −20 °C. Table 1 shows that the GC/CNT-MC/Nafion biosensors compare well with other reagentless biosensors based on the enzyme glucose dehydrogenase.

CONCLUSIONS

The chains of poly-amino-saccharide chitosan constitute a convenient scaffold for the covalent immobilization of enzymes and redox cofactors and for the dispersion of carbon nanotubes. Such a combination of properties facilitates the design and synthesis of new materials for the development of electrodes for both the electrolytic cells (e.g. biosensors) as well as galvanic cells (e.g. biofuel cells).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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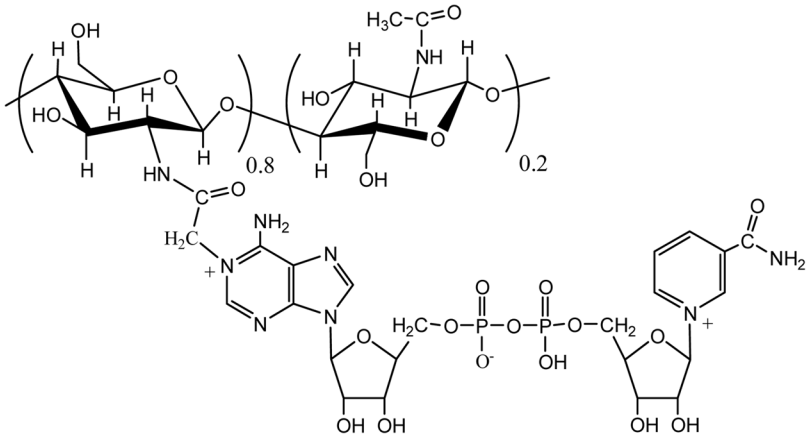

Figure 1.
Schematic structure of the polysaccharide chitosan (CHIT) with covalently attached NAD^+.

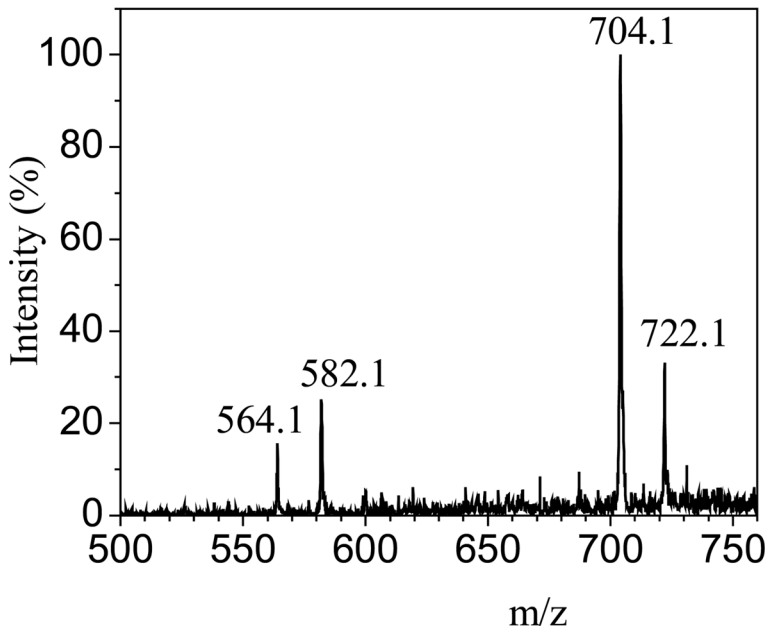

Figure 2.
Source CID mass spectrum of CHIT-NAD^+ product.

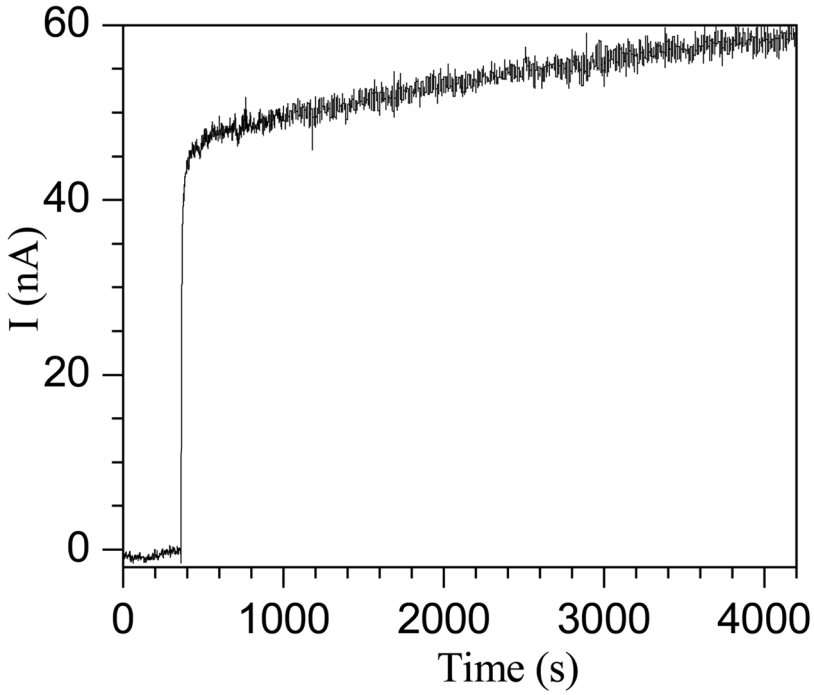

Figure 3.

Amperometric trace (E=0.40 V) recorded at a GC/CHIT-CNT electrode in a stirred solution of 40 μM CHIT-NAD^+ and 60 μg mL^{-1} GDH to which a 5 mM glucose aliquot was added at ~380 s. Background electrolyte, pH 7.40 phosphate buffer solution (0.10 M).

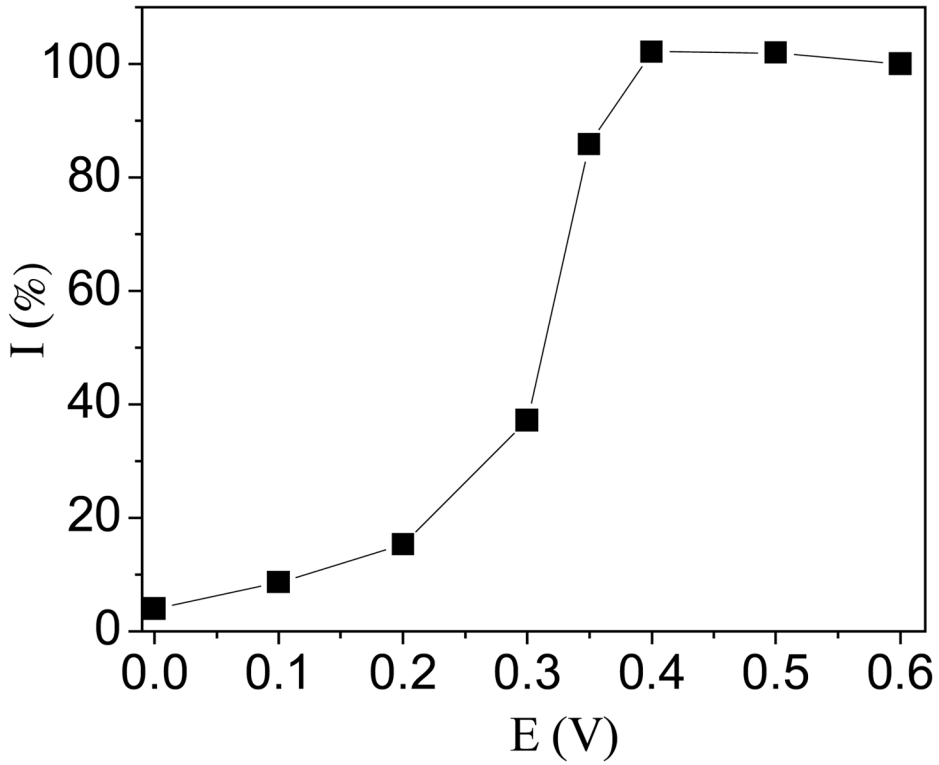

Figure 4.
Hydrodynamic voltammogram recorded at a GC/CNT-MC/Nafion electrode in a stirred solution of 5.0 mM glucose. The y-axis was normalized by assigning 100% to a current measured at 0.40 V. Background electrolyte, pH 7.40 phosphate buffer solution (0.10 M).

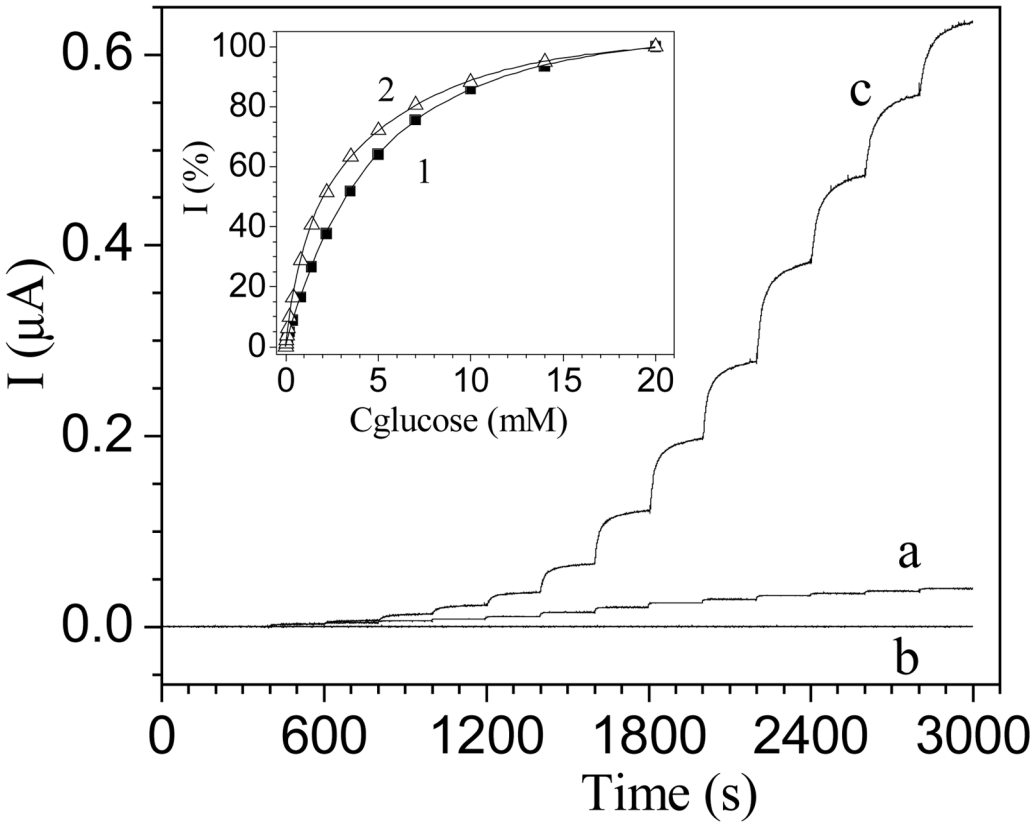

Figure 5.

Amperometric traces (E=0.40 V) recorded at (a) GC/CNT-MC, (b) 'dry' GC/CNT-MC/Nafion, and (c) 'wet' GC/CNT-MC/Nafion electrodes in stirred solutions during the additions of glucose aliquots (0.020–10 mM). Inset: calibration plots for an electrode (c) on the 5^{th} day (1) and 37^{th} day (2). Background electrolyte, pH 7.40 phosphate buffer solution (0.10 M).

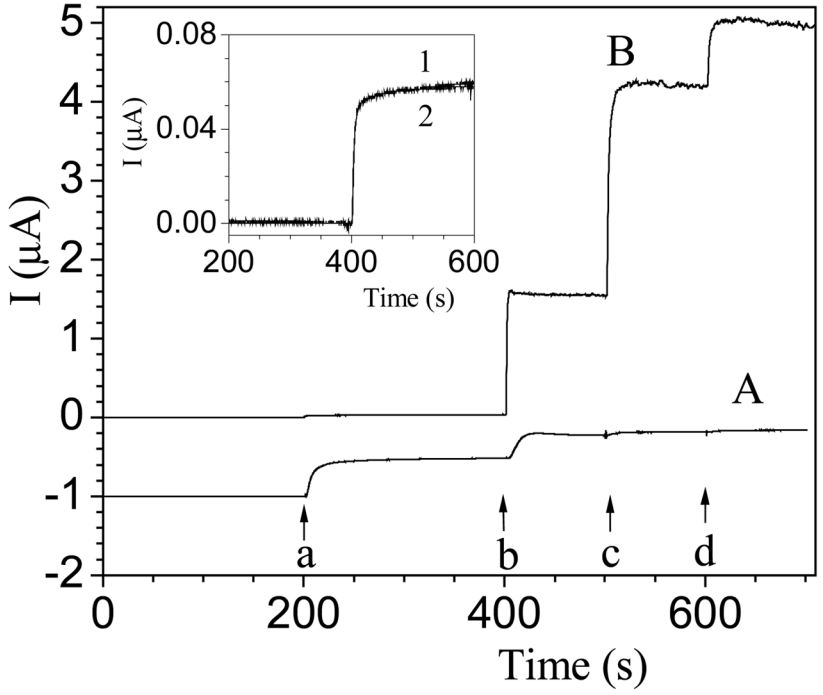

Figure 6.

Amperometric response of (A) wet GC/CNT-MC/Nafion and (B) GC/CNT-MC electrodes to (a) 5.0 mM glucose and to the interfering species (0.10 mM) such as (b) acetaminophen, (c) uric acid, and (d) ascorbic acid. Trace A was shifted downward for clarity. Inset: Amperometric response of a wet GC/CNT-MC/Nafion electrode to 0.20 mM glucose in (1) aerated solution and (2) deoxygenated solution. Background electrolyte, pH 7.40 phosphate buffer solution (0.10 M). Potential, E=0.40 V.

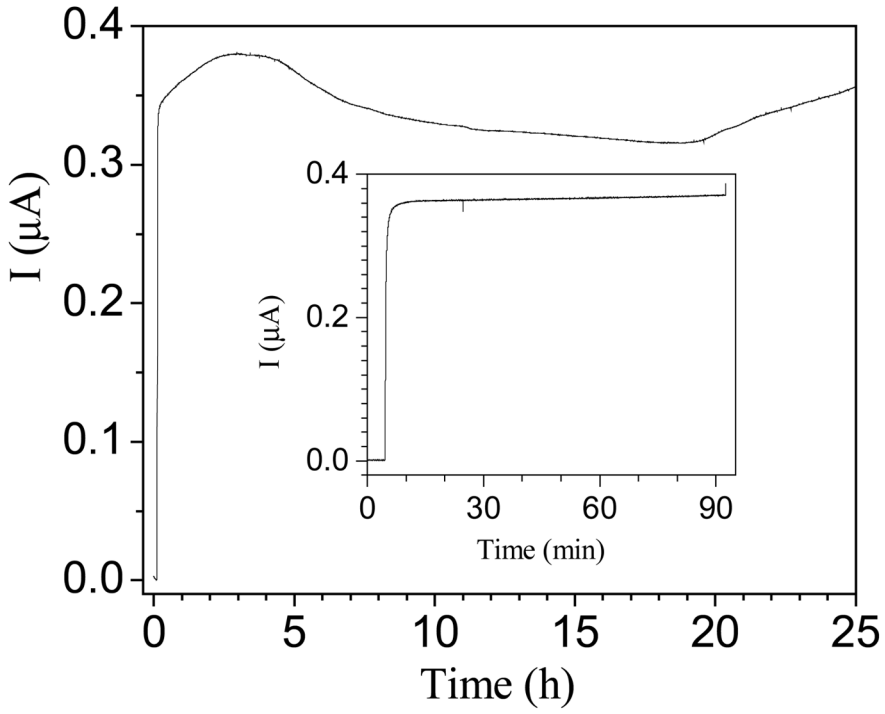

Figure 7.
Operational stability of a wet GC/CNT-MC/Nafion biosensor under continuous polarization (E=0.40 V) in a stirred solution of 5.0 mM glucose. Inset: Alternative behavior of wet GC/CNT-MC/Nafion biosensors. Background electrolyte, pH 7.40 phosphate buffer solution (0.10 M).

Table 1

Comparison of Amperometric Reagentless Glucose Biosensors Based on the Glucose Dehydrogenase/Cofactor System

sensor assembly	sensitivity, mA M^{-1} cm^{-2}	stability[a]	linear range mM	Interferences[b]	ref
GC/CNT-MC/Nafion	1.8	24 h,100%/1.5 m,64%	0.02–2.0	< 5%	*c*
GC/TFC/Ca^{2+}/PL	0.67	?	?	?	20
GC/MB/Nafion	0.49[d]	?	?	?	29
CP/PS-TBO	4.0	?	0.1–5.0	?	5
CP/PMA	0.014[d]	?/4 m (?)	5–36	?	18
CP/PMA/Nafion	0.002[d]	?	10–330	< 5%	18
CP/Osphendione	?	8 h,92%/1 m, 92%	?	?	4
CP/Ru complex	?	?/7 d (60%)	?	?	2
CP/MB	?	?/1 d, 10%	?–20	?	8
Au/Ca^{2+}/Nafion	?	10 cycles (?) /?	?	?	19

[a] operational stability (hours, h)/long-term stability (days, d; months, m). Percentages indicate signal retention;

[b] below 5 % when 5.0 mM glucose and 0.10 mM ascorbic acid and 0.10 mM uric acid were used;

[c] This work;

[d] Estimated from the data presented;

GC, glassy carbon; TFC, trinitrofluorenone-carboxylic acid; PL, polylysine; MB, Meldola Blue; CP, carbon paste; PS, modified polystyrene; TBO, Toluidine Blue O; PMA, polymethacrylate;?, not reported.