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Multilayer membranes for glucose biosensing via layer-by-layer assembly of multiwall carbon nanotubes and glucose oxidase

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Abstract

A bilayer of the polyelectrolytes poly(dimethyldiallylammonium chloride) (PDDA) and poly(sodium 4-styrenesulfonate) (PSS) was formed on a 3-mercapto-1-propanesulfonic-acid-modified Au electrode. Subsequently, multiwall carbon nanotubes (MWCNTs) wrapped by positively charged PDDA were assembled layer-by-layer with negatively charged glucose oxidase (GOx) onto the PSS-terminated bilayer. Electrochemical impedance spectroscopy and atomic force microscopy were adopted to monitor the regular growth of the PDDA-MWCNTs/GOx bilayers. Using GOx as a model enzyme, the assembled multilayer membranes showed some striking features such as the adsorbed form of GOx on individual MWCNT, uniformity, good stability, and electrocatalytic activity toward oxygen reduction. Based on the consumption of dissolved oxygen during the oxidation process of glucose catalyzed by the immobilized GOx, a sensitive amperometric biosensor was developed for the detection of glucose up to 5.0 mM with a detection limit of 58 μM. The sensitivity increased with increasing sensing layers up to five bilayers. Ascorbic acid and uric acid did not cause any interference due to the use of a low operating potential. The present method showed high reproducibility for the fabrication of carbon-nanotubes-based amperometric biosensors.

Keywords: Biosensors; Layer-by-layer; Multilayer membranes; Carbon nanotubes; Glucose; Dissolved oxygen

Carbon nanotubes (CNTs)[1] are of tremendous interest due to their attractive electronic, chemical, and mechanical properties [1,2]. Owing to the atomic structure, CNTs can behave electrically as a metal or as a semiconductor [3]. Recent studies have shown the excellent electrocatalytic activity and antifouling properties of CNTs to improve the electrochemical behaviors of nicotinamide adenine dinucleotide (NADH) [4], insulin [5], catecholamine neurotransmitters [6,7], amino acids [8], cytochrome *c* [9], nitric oxide [10], and oxygen [11,12]. The unique properties of the CNTs make them very promising in electrochemical applications, especially in electrochemical biosensors.

A major barrier for the preparation of CNTs-based biosensors is the insolubility of CNTs in most solvents. Generally, CNTs are temporarily dispersed in dimethylformamide [4,6–9] or acetone [10] and then cast on the electrodes and immobilized through solvent evaporation. The resulting CNTs layer is mechanically and electrically loose, and its application in biosensor systems is limited because the usage of organic solvents may denature biological molecules. Several strategies have been proposed to dissolve CNTs,

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[1] *Abbreviations used:* AFM, atomic force microscopy; CNTs, carbon nanotubes; EIS, electrochemical impedance spectroscopy; GOx, glucose oxidase; LBL, layer-by-layer assembly; MPS, 3-mercapto-1-propanesulfonic acid, sodium salt; MWCNT, multiwall carbon nanotubes; NADH, nicotinamide adenine dinucleotide; PBS, phosphate buffer solution; PDDA, poly(dimethyldiallylammonium chloride); PE, polyelectrolyte; PSS, poly(sodium 4-styrenesulfonate) or polystyrene sulfonate; PVP, polyvinyl pyrrolidone; RSD, relative standard deviation.

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including oxidative treatment [13], polymer wrapping [14], and sidewall functionalization [15]. Since the oxidative treatment or chemical modification impairs the physical and chemical properties of CNTs [16,17], the polymer-based solubilization of CNTs is a promising approach. Wang et al. [18] reported the solubilization of CNTs in Nafion solution and constructed a glucose biosensor based on the Nafion-solubilized CNTs. Zhang et al. [19] reported a dehydrogenase biosensor based on the solubilization of CNTs in chitosan solution. In both systems, the modification was prepared by casting CNTs solutions on the electrode surfaces. This method could not control well the properties of the resulting CNTs–polymer films. O'Connell et al. [14] reported the solubilization of CNTs in water by noncovalently associating them with linear polymer such as polyvinyl pyrrolidone (PVP) and polystyrene sulfonate (PSS). This association was characterized by tight, uniform association of the polymers with the sides of the nanotubes. The authors then demonstrated that the polymer was uniformly wrapped around the tubes rather than associated with the side walls at various points as random coils. This work opened up a new avenue to the introduction of CNTs into biologically relevant systems.

The layer-by-layer assembly (LBL) method is one of the most perspective methods for thin film deposition [20]. This technique is based on the alternate electrostatic adsorption of the negatively/positively charged individual components. This new deposition technique paves the way to fabricate sensing membranes tailored for optimum performance with regard to controlled thickness, structural morphology, and/or biocatalyst loading. It has been successfully applied to the preparation of thin films of various proteins and nanoparticles [21–23]. Recently, Mamedov et al. [24] reported the preparation of single-wall carbon nanotube/polyelectrolyte multilayer composites. The CNTs were first refluxed in 65% HNO_3 to produce the carboxylic acid groups. These negatively charged CNTs were assembled layer-by-layer with positively charged polyelectrolyte. Using glucose oxidase (GOx) as a model enzyme, this work reports a CNTs/GOx multilayer composite for biosensing application. Similar to the result of O'Connell et al. [14], this process can effectively preserve the original structure of CNTs compared to the previously acid-refluxed treatment [12,24,25]. This modification technology has opened a new avenue for the preparation of CNTs-based enzyme amperometric biosensors.

Materials and methods

Reagents

GOx (E.C. 1.1.3.4; type X-S; 179 U/mg) was purchased from Sigma; 20% PDDA (MW 100,000–200,000 g/mol) aqueous solution, 30% PSS (MW 70,000 g/mol) aqueous solution, and 3-mercapto-1-propanesulfonic acid, sodium salt (MPS) were purchased from Aldrich; multiwall carbon nanotubes (>95%, 10–20 nm diameter) were purchased from Shenzhen Nanotech Port Ltd. Co. (Shenzhen, China). All other chemicals were of analytical grade and were used as received. Twice-distilled water was used throughout.

Preparation of PDDA-wrapped MWCNTs and (PDDA-MWCNTs/GOx)$_n$ multilayer membranes

MWCNTs (1.8 mg) were solubilized in 1 ml of 5% PDDA solution with a 10-min sonication. The solution was then incubated at 50 °C for 12 h. Afterward, the solution was diluted to 5 ml with distilled water. Gold disk electrodes (0.5 mm diameter) were abraded with fine silicon carbide paper, polished carefully with 0.3- and 0.05-μm alumina slurry, and sonicated in water and absolute ethanol. The negatively charged Au/MPS surface was prepared by immersing the cleaned gold electrode in 2.0 mM MPS aqueous solution for 8 h, followed with a thorough rinse with distilled water.

The negatively charged substrate Au/MPS was first immersed in PDDA aqueous solution (2 mg/ml) and PSS aqueous solution (2 mg/ml) for 15 min, each. After being washed with distilled water, the PDDA-MWCNTs/GOx bilayers were grown on the PSS-terminated film by alternately dipping the modified gold electrode into the PDDA-MWCNTs and GOx (50 mg/ml in pH 7.4 PBS) solutions for 30 min, each. The membranes were carefully washed with distilled water after each dipping step. This sequence was repeated until the desired PDDA-MWCNTs/GOx bilayer number was obtained (Fig. 1A).

Apparatus and measurements

Amperometric and cyclic voltammetric experiments were performed on a BAS 100B electrochemical workstation (Bioanalytical System Inc., USA). All experiments were carried out using a conventional three-electrode system with the gold disk electrode as working, a platinum foil as auxiliary, and a saturated calomel electrode as reference electrodes. Unless pointed out, the experiments were carried out in air-saturated solutions. The dissolved oxygen in 0.1 M, pH 7.4, PBS was removed to prepare oxygen-free solutions by bubbling with high-purity nitrogen for 15 min.

The electrochemical impedance spectroscopy (EIS) measurements were carried out on a CHI 660 Electrochemical Workstation (CH Instruments Inc., USA) in oxygen-free solution containing 5 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl and plotted in the form of complex plane diagrams (Nyquist plots). The amplitude of the applied sine wave potential was 5 mV. The EIS was recorded with the frequency range of 1–10^5 Hz in the form of complex plane diagrams (Nyquist plots). Atomic force microscopy (AFM) images were obtained using a SPI 3800 probe station (Seiko Instruments Inc., Japan) with a Si cantilever operated in tapping mode.

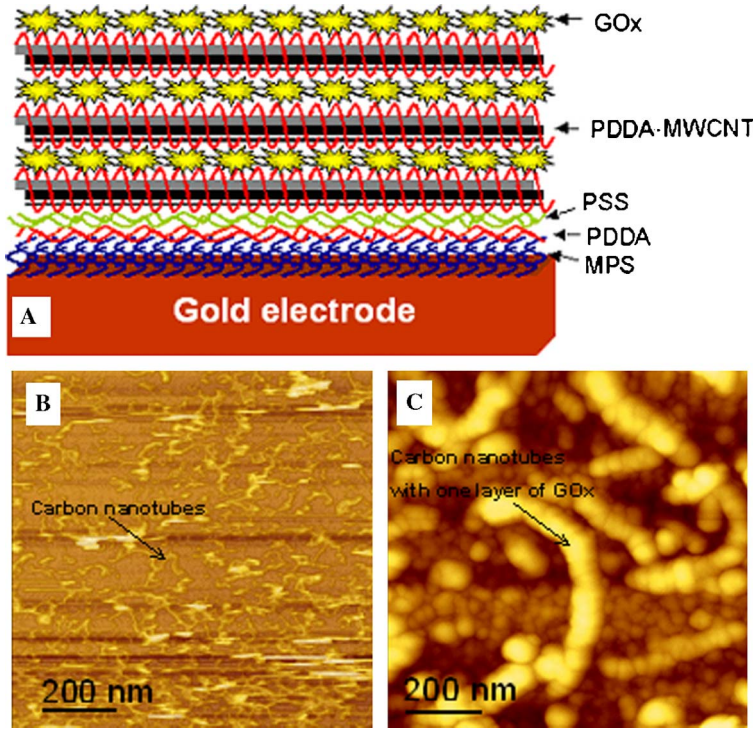

Fig. 1. (A) Schematic structure of the multilayer membranes; (B) AFM of MPS-PDDA/PSS/PDDA-MWCNTs; and (C) MPS-PDDA/PSS/PDDA-MWCNTs/GOx membranes on gold electrodes.

Results and discussion

Assembly of multilayer membranes

In pH 7.4 phosphate buffer, glucose oxidase is negatively charged. This negative charge makes it suitably incorporated in positively charged multilayer films. As shown in Fig. 1A, the polyelectrolyte (PE) PDDA could wrap on the sidewall surface of MWCNTs, resulting in positively charged PE-modified MWCNTs, PDDA-MWCNTs. Initially, a base layer of MPS was deposited onto a gold electrode surface via self-assembly. To obtain a uniform and smooth-charged surface for stable adsorption, the substrates were further immersed in PDDA and PSS solutions to form a PDDA/PSS "precursor" film. Randomly oriented carbon nanotubes could be seen from the AFM image of the obtained PDDA-MWCNTs layer (Fig. 1B). The MWCNTs covered uniformly on the entire surface of the gold electrode with an average diameter of 15 nm. The density of MWCNTs in the first bilayer was rather small, but the individual carbon nanotubes were interweaved and homogeneously integrated with PE, without any sign of phase segregation. In particular, most of MWCNTs were in the form of single nanotubes, very different from the bundles [12,24,25], indicating that this method was efficient to form a well-dispersed MWCNTs layer.

The glucose oxidase molecules could be deposited on the PDDA-MWCNTs layer by immersing the PDDA-MWCNTs-modified gold electrode in pH 7.4 PBS containing 50 mg/ml GOx. A saturated enzyme layer could be formed after 30 min. As shown in the AFM image (Fig. 1C), GOx molecules were aggregated on MWCNTs due to the large surface-to-volume ratio of MWCNTs. As a result, the nanotubes became "fat" with a diameter of about 100 nm.

The sequential repetition of the deposition of PDDA-MWCNTs and enzyme could produce a multilayer membrane with a porous structure, which would be favorable for the approach of substrates to the enzyme molecules in internal layers. In addition to the AFM characterization, evidence for the uniform deposition of PDDA-MWCNTs/GOx bilayers was obtained from electrochemical impedance data.

Electrochemical impedance characterization of the assembled membranes

EIS is a powerful tool for studying the interface properties of modified electrodes. The value of electron transfer resistance (R_{ct}) depends on the dielectric and insulating features at the electrode/electrolyte interface. Fig. 2 compares the Nyquist plots of the impedance spectroscopy at different film-modified electrodes. Here, Z'' and Z' are the real variable and the negative value of the imaginary variable of impedance, respectively. On the precursor film, the plot showed the greatest semicircle corresponding to the largest electron transfer resistance, due to the electrostatic repulsion between negatively charged surface and probe molecule, $Fe(CN)_6^{3-/4-}$ (curve a). After adsorption of PDDA-MWCNTs, the surface became positively charged,

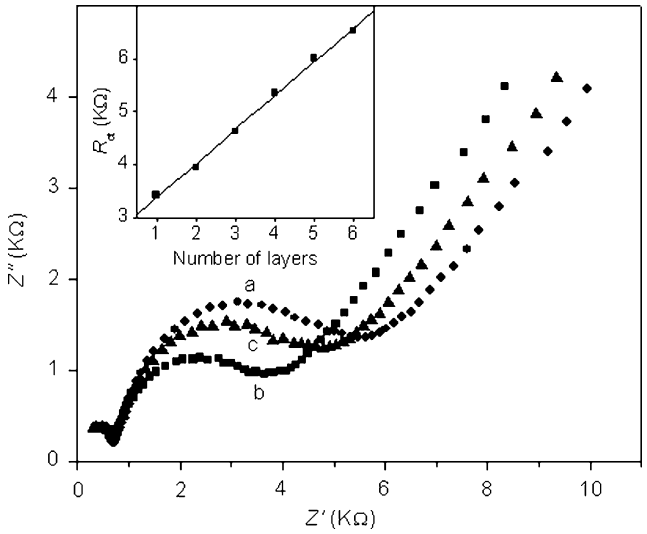

Fig. 2. Nyquist plots of MPS-PDDA/PSS (a), MPS-PDDA/PSS/PDDA-MWCNTs (b), and MPS-PDDA/PSS/PDDA-MWCNTs/GOx, (c)-modified gold electrodes. Inset: Plot of electron transfer resistance of MPS-PDDA/PSS/{PDDA-MWCNTs/GOx}$_n$ modified gold electrode versus the bilayer number n.

resulting in a lowest electron transfer resistance (curve b). With further adsorption of GOx, the surface turned negatively charged again, and the enzyme with negative charges also blocked the access of the probe molecules to the electrode surface, resulting in the increase of the electron transfer resistance (curve c). However, the value of R_{ct} was less than that at the precursor film-modified electrode, even if the film thickness became larger after assembly of GOx. This indicated the contribution of MWCNTs even though they were wrapped by PDDA. MWCNTs promoted the electron transfer of the probe molecules and increased the electrode surface.

Upon the stepwise PDDA-MWCNTs/GOx bilayers formation, the membranes on the electrode became much thicker. Therefore, the electron transfer resistance increased with the increasing bilayer number [26]. A good linear relationship between R_{ct} and bilayer number was observed (inset, Fig. 2), indicating a well-behaved LBL assembly process.

Electrocatalytic activity of the assembled membranes toward oxygen reduction

The freshly polished gold electrode in 0.1 M pH 7.4 PBS exhibited an irreversible reduction peak of dissolved oxygen at around −0.30 V at 50 mV/s (curve a, Fig. 3A), which was documented to be a two-electron ($2e^-$) reduction process to produce hydrogen peroxide [27–29]. This process was greatly blocked by the PDDA/PSS "precursor" film, resulting in an ill-defined peak (curve b, Fig. 3A). With introduction of five PDDA-MWCNTs/GOx bilayers on the electrode surface, a well-defined reduction peak appeared at −0.21 V again (curve b, Fig. 3B). After removal of the dissolved oxygen, this response disappeared, indicating the reduction of dissolved oxygen (curve a, Fig. 3B). Compared to curve b of Fig. 3A, the peak shape was greatly improved, and the reduction peak potential was even more positive than that at bare gold electrode, indicating an electrocatalytic behavior of the membranes to oxygen reduction due to the presence of carbon nanotubes [11,12,30]. With increasing scan rate, the peak current increased and the peak potential shifted negatively (not shown). The peak current was proportional to the square root of the scan rate. Thus the reduction process involved the diffusion of O_2 to electrode surface, which could be expressed as follows [11]:

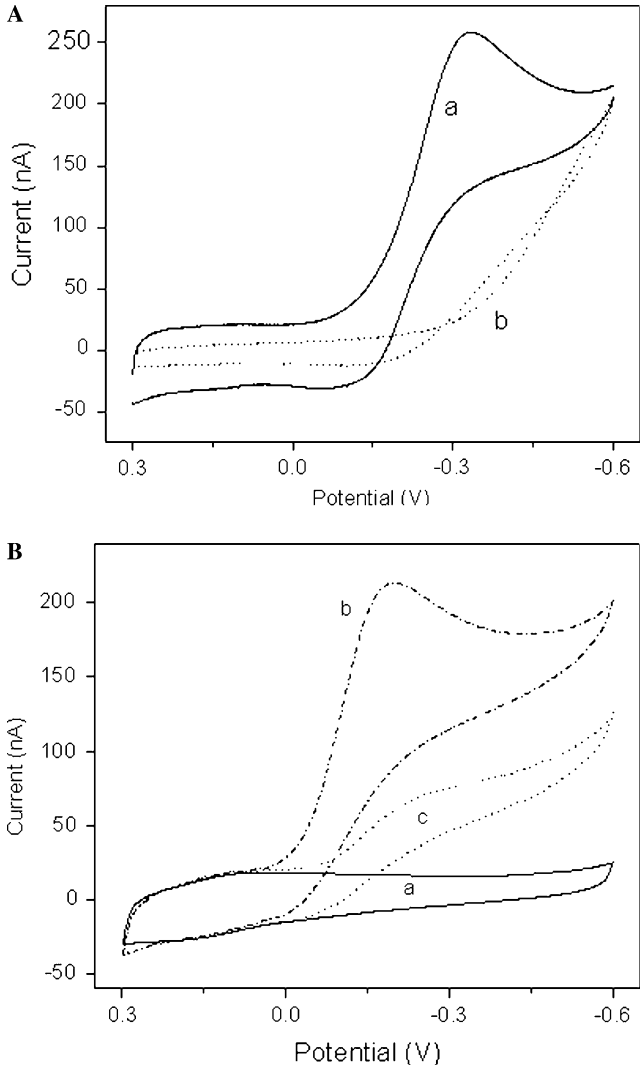

Fig. 3. Cyclic voltammograms of (A) 0.1 M, pH 7.4, PBS at freshly polished (a) and MPS-PDDA/PSS-modified gold electrode (b) in the presence of dissolved oxygen and (B) MPS-PDDA/PSS/{PDDA-MWCNTs/GOx}$_5$-modified gold electrode in 0.1 M, pH 7.4, deoxygenated PBS (a), air-saturated PBS (b), and air-saturated PBS plus 10 mM glucose (c) at 0.05 V/s.

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2. \quad (1)$$

Multilayer membranes as sensing element for glucose detection

The oxidation of glucose in the presence of glucose oxidase follows Eq. (2):

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{Gox}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2. \quad (2)$$

The consumption of dissolved oxygen during glucose oxidation led to the decrease of O_2 concentration; thus the electrochemical response of dissolved oxygen decreased (curve c, Fig. 3B). Upon addition of 10 mM glucose to air-saturated PBS, the reduction peak current of dissolved oxygen decreased from 179 to 42 nA at 50 mV/s. Thus, this could be developed for the amperometric determination of glucose.

Considering the best signal-to-noise ratio, the amperometric measurements were performed in a stirred 0.1 M pH 7.4 PBS at an applied potential of −0.2 V. The amperometric response and the sensitivity of the fabricated biosensors to glucose were related to the bilayer number of PDDA-MWCNTs/GOx. As shown in Fig. 4, the sensitivity (the obtained linear slope divided by the electrode area) of the biosensor increased with increasing bilayer number up to 5 and then decreased greatly. This could be attributed to the blocked diffusion of glucose to the entrapped enzyme through the thicker films [31]. Thus, the {PDDA-MWCNTs/GOx}$_5$ membranes-modified gold electrodes were used as glucose biosensors, and the maximum sensitivity was measured to be 5.6 μA/(mM cm^2). The sensitivity was higher than that of 3.9 μA/(mM cm^2) at {PDDA/gold nanoparticles/PDDA/GOx}$_n$ membranes-based glucose biosensors [23] and 1.0 μA/(mM cm^2) at carbon-nanotube-modified basal plane pyrolytic graphite electrode [32].

The typical steady state amperometric response to the successive addition of 1.0 mM glucose is shown in Fig. 5.

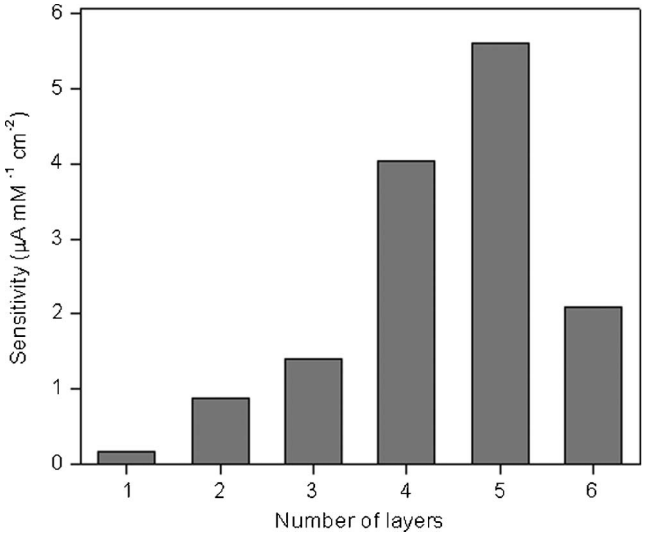

Fig. 4. Sensitivity of MPS-PDDA/PSS/{PDDA-MWCNTs/GOx}$_n$-modified gold electrode to glucose vs the number of bilayers.

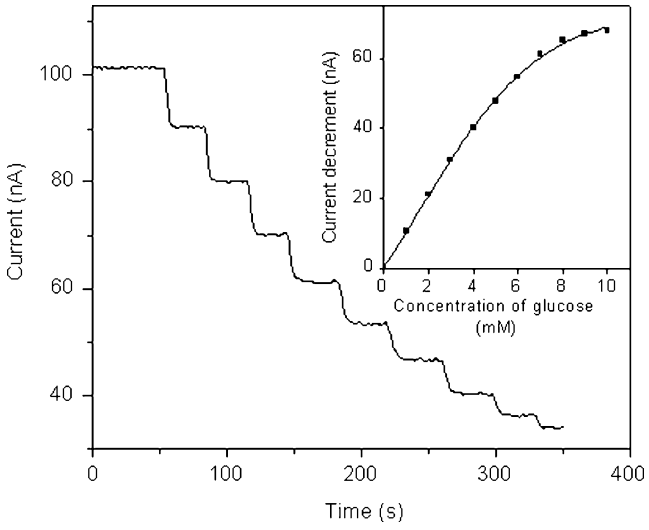

Fig. 5. Amperometric response of MPS-PDDA/PSS/{PDDA-MWCNTs/GOx}$_5$-modified gold electrode at an applied potential of −0.2 V to successive addition of 1 mM glucose in a stirred 0.1 M, pH 7.4, PBS in the presence of dissolved oxygen. Inset: Calibration curve.

For each injection of 2 μL of 1 M glucose in 2 mL PBS, a sharp decrease in the current was observed. The response reached 95% steady state value within 8 s. The response decreased with increasing glucose concentration (inset, Fig. 5). The linear calibration range was extended up to 5.0 mM ($R = 0.999$, $n = 6$), compared with that of 2.0 mM for the oxygen-sensitive glucose biosensor [30,33]. Such extended linear range might arise from two effects—oxygen reduction promoted by MWCNTs and the increased oxygen content around the electrode surface with increasing bilayer number. The linear range was considered to be useful for the normal glucose concentration in blood serum of around 4.6 mM [34]. The detection limit was estimated to be 58 μM at a signal-to-noise ratio of 3.

The effects of common interfering species on the biosensor response were examined. Fig. 6 shows the effects of interfering species, including ascorbic acid and uric acid, when a detection potential of −0.20 V is employed. The injection of 1.0 mM glucose caused an immediate decrease of the reduction current, while subsequent injection of ascorbic acid and uric acid did not cause any interference on the current response. The use of a low operating potential greatly reduced the interference; thus, highly selective response to glucose was obtained without any mediator or perm-selective membrane. This was an advantage of the proposed biosensor over those reported previously [23,35–37].

As control, an electrode modified with five PDDA/GOx bilayers without MWCNTs was prepared. This electrode did not display any amperometric response to glucose at −0.2 V. At applied potentials more negative than −0.3 V, a weak amperometric response was observed, but it was inapplicable to the detection of glucose. Therefore, MWCNTs decreased the overpotential of oxygen reduction and favored the detection of glucose at −0.2 V. Thus, the

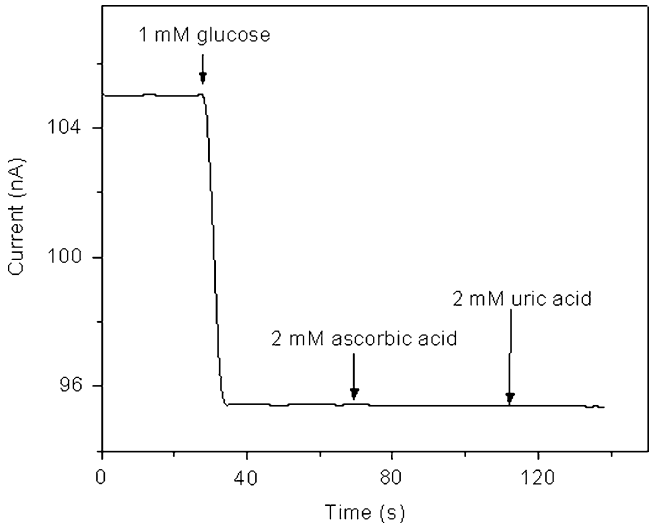

Fig. 6. Effect of interfering species on the biosensor response at an applied potential of −0.2 V in a stirred 0.1 M, pH 7.4, PBS in the presence of dissolved oxygen.

presence of MWCNTs was an important factor for the successful application of this biosensor.

The reproducibility and storage stability of the biosensor were also examined. The relative standard deviation (RSD) of the biosensor response to 1.0 mM glucose was 3.2% for 11 successive measurements. The RSD for detection of 1.0 mM glucose with four sensors prepared under the same conditions was 3.6%. When the biosensor was stored dry at 4 °C and measured at intervals of 1 week, it retained about 82% of its original sensitivity after 5 weeks. On the other hand, the response to dissolved oxygen had no apparent decrease, indicating that the assembled MWCNTs were stable.

Determination of glucose in human blood serum

The response of the biosensor to the glucose in human blood serum was investigated. Five serum samples obtained from hospitalized patients were analyzed. The results were matched with referenced values obtained by the automated standard colorimetric technique in the hospital. Table 1 shows the comparison of the determined values and the referenced values. Analytical recoveries of the glucose solution added to blood serum samples were from 97.0 to 104.0%.

Table 1
Determination of glucose in blood serum samples

No.	Known value (mM)	Determined value (mM)	Relative deviation (%)	Glucose added (mM)	Glucose founded (mM)	Recovery (%)
1	4.7	4.8	2.1	2	1.98	99.0
2	4.9	5.1	4.1	2	2.06	103.0
3	4.6	4.6	0	2	2.08	104.0
4	4.9	5.0	2.0	2	2.03	101.5
5	5.0	4.8	−4.2	2	1.94	97.0

Conclusions

A uniform and stable multilayer membrane of MWCNTs and enzyme can be prepared through the layer-by-layer assembly technique after modification of MWCNTs with PDDA. This membrane shows a porous structure and the assembled MWCNTs show an electrocatalytic activity to the reduction of dissolved oxygen. Based on the competition between the electrochemical reduction of dissolved oxygen and the oxidation of glucose by dissolved oxygen catalyzed with immobilized GOx, the electrocatalytic response can be used for the detection of glucose at a relatively low applied potential, at which the interference of ascorbic acid and uric acid can be excluded. This modification technology extends the application of CNTs in enzyme amperometric biosensors.

Acknowledgments

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