

Full Paper

Eliminating the Interference of Ascorbic Acid and Uric Acid to the Amperometric Glucose Biosensor by Cation Exchangers Membrane and Size Exclusion Membrane

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Received: May 19, 2005

Accepted: August 4, 2005

Abstract

The characteristics of a multiuse planar amperometric biosensor modified with Nafion and/or polyion membrane were investigated. A new enzyme immobilization process was proposed, in which the polyvinyl alcohol bearing a styrylpyridinium (SbQ)/glucose oxidase composite was treated with glutaraldehyde vapor prior to the photo-crosslinking reaction. The resulting planar enzyme electrode remains active for at least 150 days. Compared with poly-L-lysine/poly(4-stryenesulfonate) polyion complex membrane the Nafion membrane showed marked effect to reduce the electrochemical response of the modified planar enzyme electrode to the biological interferents, such as ascorbic acid and uric acid. Furthermore, Nafion membrane can effectively restrict the oxidized anionic interferent to adhere on its surface, thereby the fouling of the electrode was avoided.

Keywords: PVA-SbQ, Nafion, Planar electrode, Amperometric biosensor, Polyion

DOI: 10.1002/elan.200503359

1. Introduction

Recent trends in research and development of sensing systems have put increased emphasis on the development of small, portable and battery-operated instruments suited for on-site decentralized biomedical diagnosis and industrial-analysis [1–3]. The electrochemical instruments should be ideal for this purpose, because they are potentially compact, inexpensive, robust, and versatile [4]. Because planar electrodes are inexpensive fabrication and easily mass-produced [5–8], the planar electrode-based sensors have been widely used for the fabrication of commercialized sensors for measuring blood glucose and other electrolytes in the past few years. Furthermore, planar electrodes have also been introduced in the fabrication of portable cartridge-based critical-care systems. However, planar sensors have a shorter life-span than that of the traditional three-dimensional sensors [9–11]. Moreover, the planar glucose sensor exhibits a problem of absorbing oxidative products on the surface of the electrode in the attempt of continuous detection of glucose [12]. The adsorbing of oxidized electroactive species on the surface of electrode may shorten the life-span of a planar sensor, which is a serious problem for the multiuse or continuous analysis purpose.

Recently, a highly sensitive amperometric biosensor for glutamate has been fabricated by immobilizing enzyme in a photo-crosslinkable polymer, polyvinyl alcohol bearing a styrylpyridinium (PVA-SbQ), membrane on a palladium-

deposited screen-printed carbon electrode [13]. The polymer was previously reported to be suitable for fabrication of a thin enzyme membrane (about 1 μm thick) [14]. Enzyme can be immobilized in the PVA-SbQ matrix with high surface density and retain their functional characteristics to a large extent for several months upon repetition of wetting and drying [15]. Moreover, enzymes can be immobilized in this polymer using photolithography techniques [16], which can be adapted to mass production using ordinary screen-printing or semiconductor-fabrication processes on a planar electrode [14, 17, 18].

Strong electrochemical interference from oxidizable species, such as ascorbic acid and uric acid, in the biological samples exposes a serious problem for the practical operation of amperometric biosensors with a working potential of 0.4 V or higher [19]. For example, electrochemical oxidation of ascorbic acid (AA) generates the dehydroascorbic acid (DAA), with the loss of two electrons and the consequent loss of hydrogen ions. One way to solve this problem is to modify the electrode surface with a permselective membrane. A variety of polymer membranes have been reported to be useful for eliminating interferents [20–24]. These polymers show permselective properties based on size exclusion (e.g. poly-L-lysine and poly(4-stryenesulfonate) membrane) and/or charge interaction between solutes and the membrane (e.g., Nafion). The polymeric structure of Nafion contains a hydrophobic ($-\text{CF}_2-\text{CF}_2-$) backbone and hydrophilic sulfonic acid

groups ($-\text{SO}_3\text{H}$) which contributes its electron-withdrawing effect. Although Nafion membrane, a fluorine-containing material, is difficult to make another membrane adhere to it [23], the high viscosity of PVA-SbQ makes it possible to encapsulate the Nafion-covered electrode strip with an immobilized enzyme.

In the study, biosensors were fabricated on the Nafion- and polyion-modified palladium strips. Interestingly, we found that Nafion membrane is capable of eliminating the electrochemical interferences of oxidative species and avoiding fouling from reaching the electrode during the testing of biological samples. The polyion membrane-modified palladium strip, however, exhibited limited effect to reduce such interferences.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA), glucose, glutaraldehyde, poly-L-lysine hydrobromide (molecular weight, 100 000) and glucose oxidase (GOD, EC 1.1.3.4) were purchased from Sigma Chemical Co. (St. Louis, USA). Nafion (perfluorinated ion-exchange resin, 5% w/v solution in lower alcohol/water), was purchased from Aldrich (Steinheim, Germany) and used as supplied. Poly(sodium 4-styrenesulfonate) (molecular weight, 70 000) was purchased from Aldrich (Milwaukee, WI). PVA-SbQ (degree of polymerization 1700, degree of saponification 87%) was obtained from Toyo Gosei Kogyo Chemical (Tokyo, Japan).

2.2. Electrode Modification

A commercial glucose test strip (Boehringer-Mannheim) consisting of two palladium strip electrodes was used [4]. The palladium connecting strip of the commercial electrode were used as electrode by cutting off the enzyme loaded pad. The prepared palladium strips were then modified and connected to a laboratory-built potentiostat as the work and counter electrodes. The Nafion (5% w/v solution in lower alcohol/water) and/or polyion membrane was then added onto a connecting area of the commercial electrode.

2.3. Enzyme Immobilization

Prior to enzyme immobilization, the working area of an electrode was covered with Nafion coating and/or polyion membrane. The Nafion was used as supplied, 0.5 μL Nafion was carefully spread on the active surface and dried for 8 h at room temperature. The polyion membrane was modified as follows: The polyion membrane was prepared by spreading 1.0 μL poly-L-lysine (25 mM in the monomer unit) on the connecting area of the commercial electrode to form a thin film. The electrodes were then allowed to dry at room temperature for 4 h. Subsequently, the poly-L-lysine modi-

fied electrode was further coated with a thin layer of poly(4-styrenesulfonate) (25 mM in the monomer unit, 1.0 μL). The electrodes were allowed to dry for 4 h at the room temperature and then placed in a vacuum chamber. The chamber was evacuated at a base pressure of ca. 1 Pa for one further hour [19].

After that, the GOD was immobilized by a combination of PVA-SbQ photocross-linking and glutaraldehyde exposure. A mixture of 50 mg PVA-SbQ, 150 mg L-GOD (500 U mL^{-1}) was prepared and 1 μL of this mixture was deposited onto the active area of the electrode, the sensor was then placed in a dark sealed box containing glutaraldehyde vapor. The box was kept at 4 °C for 8 h to allow crosslinking to complete. Subsequently, the sensor was exposed to UV light for 25 min, followed by immersing the sensor in the phosphate buffer (100 mM, pH 7.0) for one hour. The electrodes prepared were then stored at 4 °C under dry dark conditions. The electrodes were immersed in the phosphate buffer for one hour before use.

2.4. Apparatus and Procedure

Amperometric measurements were performed using a laboratory-built potentiostat. Input and output signals from the potentiostat were coupled to a PC (Pentium 600 MHz) using a peripheral interface card (AT-MIO-16E, National Instruments, Austin, Texas, USA). The interface card consisted of a 16-channel analog-to-digital (A/D) converter (12 bit) and a 2-channel digital-to-analog (D/A) converter (12 bit). Voltage output, data display and recording were programmed using the LabVIEW 6.1 software package (National Instruments, Austin, Texas, USA). All measurements were taken with a three-electrode system using an Ag/AgCl electrode as a reference and a modified commercially palladium strip (4.8 mm^2) as the working and counter electrodes. The amperometric measurement was performed at 0.5 V vs. Ag/AgCl. The working solution was 9.9 mL PBS buffer (pH 7.0) in a cylindrical cell, with temperature controlled using a thermostat. All experiments were carried out at 37 °C. For the amperometric measurement of glucose, 0.1 mL of glucose solution was injected into the test solution using a microsyringe when a steady state of the testing-system had been obtained. The baseline current was measured, and then, following the injection of glucose, the response current was displayed and simultaneously logged by the computer until a steady state was achieved. Magnetic stirring during the operation was used to ensure the homogeneity of the solution. The difference between the baseline and the steady-state current was used to calculate the concentration of glucose.

3. Results and Discussion

Figure 1 illustrates the simple diagram of fabricated glucose biosensor with PVA-SbQ/GOD composite on a permselective membrane-modified Pd electrode. PVA-SbQ is a polyvinyl alcohol bearing a styrylpyridinium (SbQ) residue

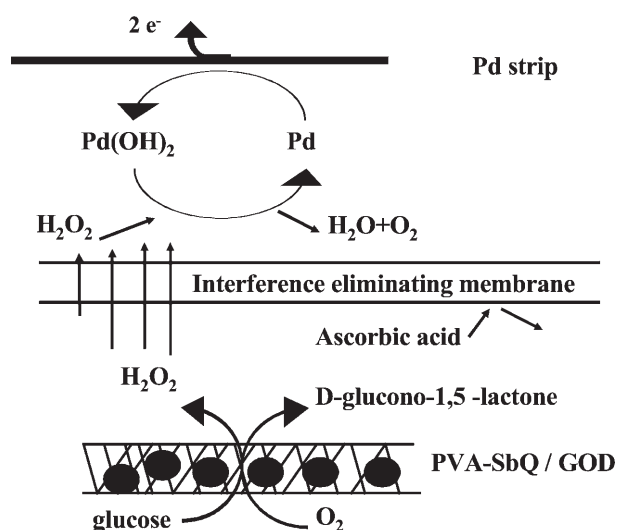


Fig. 1. Schematic diagram illustrating the redox reactions occurred on the PVA-SbQ/GOD/permselective membrane-modified Pd electrode.

as its photosensitive group. The cross-linking of the polymer occurs when UV radiation induces cyclodimerization of styrylpyridinium groups and produces porous network-like matrix to entrap enzymes [18,25,26]. A rapid and highly sensitive measurement of analyte can be achieved, because substrate can diffuse quickly through the porous PVA-SbQ thin layer to the entrapped enzymes. The entrapped GOD can catalyze the conversion of glucose into D-glucono-1, 5-lactone and hydrogen peroxide. The oxidation of hydrogen peroxide is then catalyzed by Pd under a given potential and generates an anodic current, which is used to monitor the oxidation of glucose. Uric acid and ascorbic acid are commonly found in biological fluids of humans. They may cause severe interference on an electrochemical sensor system. In this paper, two permselective membranes were used to modify the Pd-deposited electrode to find out whether they were suited to diminish the interference response of these electroactive species.

3.1. Optimization of the Photopolymer Composition on Sensor Response

Using PVA-SbQ membrane as an enzyme immobilizing matrix in the fabrication of biosensors provides two advantages: (1) The formation of network-like polymeric matrix of PVA-SbQ can be carried out under a relative mild condition that may reduce the damage to the entrapped enzymes; (2) Enzymes in this polymer can be easily immobilized on a planar electrode using photolithography techniques [16], which can be adapted to mass production of various biosensors using ordinary screen-printing or semiconductor-fabrication processes [15, 17]. However, the level of cationic SbQ groups must be carefully controlled to prevent the deterioration of PVA-SbQ matrix. It has been shown that the activity of encapsulated enzymes might be

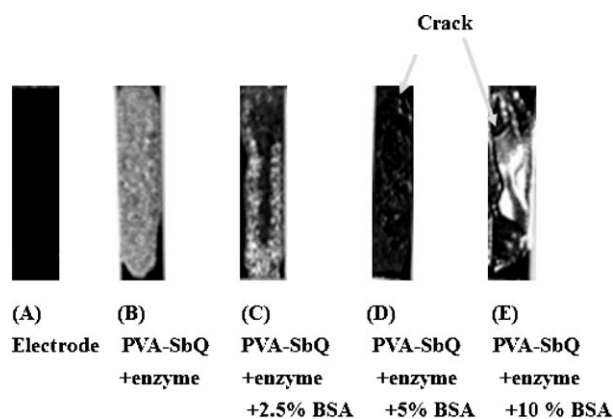


Fig. 2. The microscopic images of the PVA-SbQ/enzyme composite without or with various amounts of BSA. The mechanic cracking on the electrode was marked by an arrow.

reduced with the high percentage of cationic SbQ groups (e.g., 14 mol% to the vinyl polymer backbone). It is postulated that the reduction of the enzymatic activity may be because of the coulombic interaction between the biomolecule and the cationic SbQ group [16]. In contrast, with low percentage of SbQ, the entrapped enzymes may easily escape from the PVA-SbQ matrix [13, 25]. It has been reported that biomolecules with molecular weight greater than 6.5×10^4 , such as glucose oxidase (M. W. $\approx 1.6 \times 10^5$), can be efficiently retained in a PVA-SbQ matrix consisting 0.9 mol% SbQ group [25].

However, the PVA-SbQ membrane at 0.90 mol% SbQ content may not be so stable and tend to be dissolved in the aqueous solution [25]. Therefore, the content of SbQ in vinyl polymer was controlled at 1.10 mol% in following experiments. More than 90% vinyl polymer membrane was insoluble with 1.10 mol% SbQ as describe previously [25]. Although 1.10 mol% SbQ in vinyl polymer is enough to retain most of enzymes during immobilization, it is still possible that enzyme may escape from the pores of PVA-SbQ matrix [25]. The GOD entrapped in PVA-SbQ reagent was then further fixed by a bifunctional reagent glutaraldehyde prior to the photocrosslinking by UV [13, 25]. Glutaraldehyde can form Schiff base-type covalent bonds with primary amino groups in the target compounds and in proteins. These enzyme/glutaraldehyde/enzyme cross-linked complexes can prevent the leakage of entrapped enzyme from PVA-SbQ matrix. Although GOD could be effectively entrapped in the vinyl polymer consisting 1.10 mol% SbQ group, the activity of entrapped enzymes might be inactivated because of coulombic interaction between the enzyme and SbQ group [16, 18]. BSA has been suggested previously that it might interact with the plus-charged SbQ group and decrease the coulombic interaction between the enzyme and the SbQ group [18]. Surprisingly, we found the addition of BSA in the PVA-SbQ/GOD composite reduces the response of enzyme electrode to glucose. The reduction effect of BSA may be due to the effect of diffusion limit associated with exclusion of substrate from the interior of the PVA-SbQ matrix. More-

over, the membrane tends to crack when the enzyme solution contains a high percentage of BSA (Fig. 2).

In conclusion, a modified PVA-SbQ-based protein immobilizing process for the fabrication of amperometric biosensors is proposed in this study. A treatment of PVA-SbQ/GOD composite on the electrode with glutaraldehyde vapor prior to the photocrosslinking reaction seems essential to prevent the leakage of entrapped enzyme and yet retain most of activity of entrapped enzymes. Care must be taken to remove ammonium sulfate from enzyme, because inorganic salt may cause the separation of the polymer in an aqueous solution. The fabricated PVA-SbQ/GOD electrode exhibited remarkable long-term stability when stored under dry dark condition for 150 days. Most of the functional characteristics could be restored within five minutes (wet-up times) of repeated wetting and drying (data not shown). The fabricated enzyme electrode is superior in stability compared with most commercialized membrane-covered electrodes which have a shelf-life of about ten days [9–11] and was comparable to conventional three-dimensional electrodes.

3.2. Determination of Glucose Concentration

The electrochemical properties of fabricated glucose biosensor consisting PVA-SbQ/GOD composite on a permselective membrane-modified electrode were investigated. After obtaining a steady background current, a given volume of the glucose sample solution was injected into the test solution using a microsyringe. Magnetic stirring during the operation was used to ensure the homogeneity of the solution. Soon after, the amperometric current started to increase, indicating the production of hydrogen peroxide. Figure 3A shows the current–time response of the enzyme electrode under the optimized experimental conditions. The enzyme electrode exhibited a linear calibration range in concentrations of glucose from 50 μM to 250 mM (Fig. 3B), with a slope of 40.95 nA/mM and a correlation coefficient of 0.9929 ($n = 12$). The detection limit of glucose was 50 μM ($S/N = 3$). The concentration of glucose in normal sera is around 5 mM. The dynamic range of the presented enzyme electrode can certainly fit the need for determination of blood samples in a clinic laboratory. The shapes of the current–time curve depend on the concentration of glucose. In the case of high glucose concentration, the current sharply increased and quickly achieved a steady-state current. As shown in Figure 4B, a rapid response was achieved upon adding 5 mM glucose into the reaction cell. The current of the sensor started to increase about 3 seconds after injection of glucose and reached a steady-state current within 17 seconds. In contrast, as the enzyme glucose concentration decreased, the current–time curves showed S-shaped. The maximum was shifted to the direction of longer time values and a several seconds gate time elapsed before the current began increasing linearly with the extension of reaction time. As shown in Figure 4A, the current–time curve upon adding 1 mM glucose into the

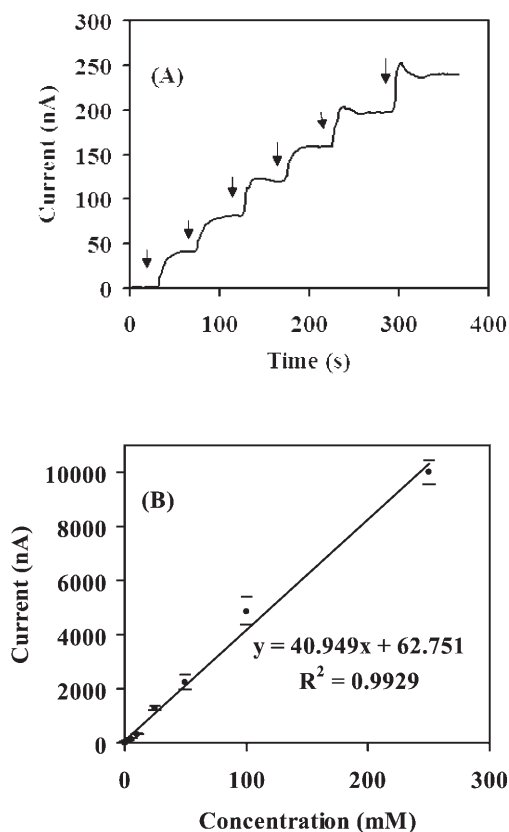


Fig. 3. A) Amperometric response of planar enzyme electrode with successive additions of 1 mM glucose. B) Calibration graph of glucose on the amperometric sensor. The arrows indicated the time points of substrate addition. Measurements were carried out at 37 °C in 100 mM sodium phosphate buffer (pH 7.0) under stirring. The polarizing potential applied to the sensor was 0.5 V vs. Ag/AgCl.

reaction cell. The 95 % steady-state current response time increased to 25 seconds. It is possible that, at high concentrations, the access of glucose was quickly catalyzed in the outmost layer of the enzyme membrane to hydrogen peroxide, which was immediately transferred to the electrode surface for further electrochemical reaction. Therefore, the current increased immediately after the glucose was injected. However, at low concentrations, the reaction rate is controlled by the mass-transfer rate of glucose and oxygen [27]. Hence, the electrochemical reaction of hydrogen peroxide is slow at low glucose concentration.

3.3. Interference Elimination from Other Electrochemically Active Substances

The use of enzyme-based amperometric biosensors for determining analytes in complex media, such as biological fluids, poses a serious problem during clinical diagnosis, of which the electroactive species, including ascorbic acid (AA) and uric acid (UA), giving a marked Faradic response at the giving potential [12]. One way to eliminate the interference of electroactive species is to modify the

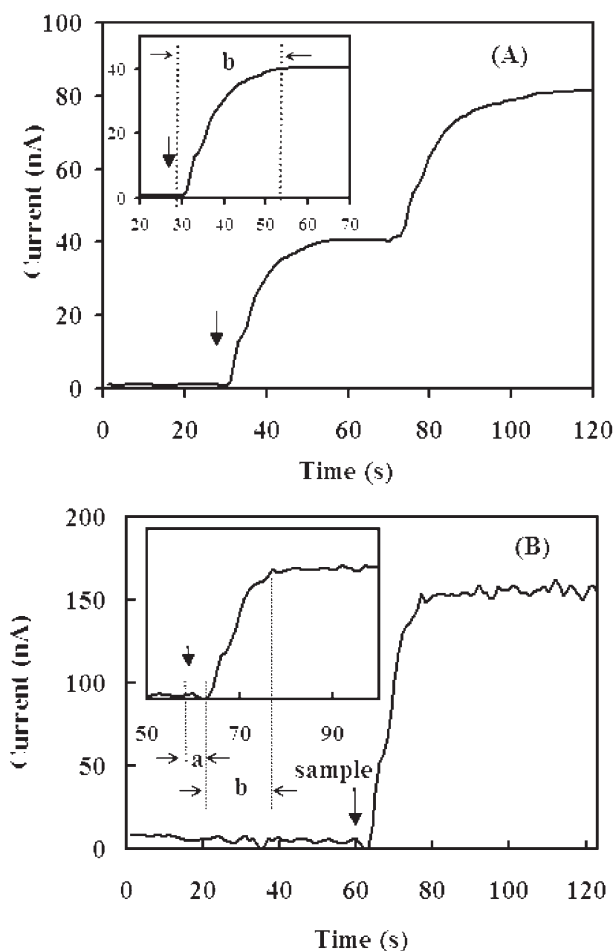


Fig. 4. Amperometric response of the PVA-SbQ/GOD/Pd biosensor to 1 mM (A) and 5 mM glucose (B). The arrows indicated the time points of substrate addition. Measurements were carried out at 37 °C in 100 mM sodium phosphate buffer (pH 7.0) under stirring. The polarizing potential applied to the sensor was 0.5 V (vs. Ag/AgCl).

electrode surface with permselective membrane. In the presented study, Nafion and polyion membranes were investigated to determine whether they could diminish the interference of AA and UA. As shown in Figure 5, Nafion could effectively reduce the electrochemical response of enzyme electrode to AA and UA; whereas polyion film only had a small effect. With conventional Pd/PVA-SbQ/GOD electrode the electrochemical responses for 5 mM glucose, 5 mM AA and 5 mM UA are 159, 227 and 202 nA, respectively (Table 1). The interference levels (the ration of electrochemical response to interferent to the electrochemical response of glucose) for AA and UA were calculated as 1.43 and 1.27, respectively. Interestingly, the interference levels to same concentration of AA and UA were markedly decreased with the Nafion-modified enzyme electrode (0.24 for AA and 0.21 for UA). The interference levels of AA and UA, however, were partially reduced with polyion as a permselective membrane (0.91 for AA and 0.65 for UA). Comparing with polyion membrane, Nafion shows significant effect to eliminate the interference of AA and

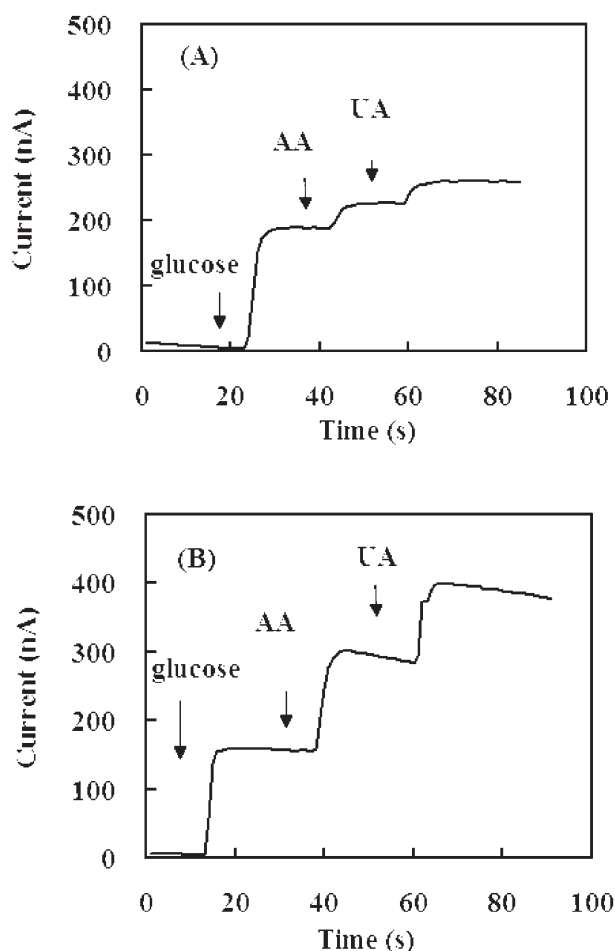


Fig. 5. The effect of Nafion (A) and polyion membrane (B) to the electrochemical responses to 5.0 mM glucose, 5.0 mM ascorbic acid (AA) and 5.0 mM uric acid (UA).

UA. Nafion is a perfluorinated cation exchange polymer with a hydrophobic perfluoro-backbone and pendant sulfonic acid groups, which allow the permeation of hydrogen peroxide and yet restrict the passage of anions (e.g., ascorbic acid and uric acid) across the membrane. It is postulated that Nafion, an anionic polymer, can effectively restrict the anionic interferent to adhere on its surface (Fig. 6A); thereby less fouling of oxidized ascorbic acid was found on the Nafion modified electrode. The polyion membrane was generated by codeposit of poly-L-lysine and poly (4-styrenesulfonate), which can be used as an ultrafilter that shows a molecular weight cut-off of ca. 100. Although, polyion membrane was demonstrated previously to effectively eliminate the anionic interferents [19], it exhibited limited effect in the present study. It is apparent that polyion membrane cannot restrict the anionic interferent to adhere on its surface (Fig. 6B). The concentration of glucose in normal sera is around 5 mM and the concentrations of ascorbic acid and uric acid are near upper limits of their physiological concentration range are 0.05 and 0.5 mM, respectively [19]. For measuring glucose in sera with the present Nafion modified planar electrode, the current change caused by the total ascorbic acid and uric acid is

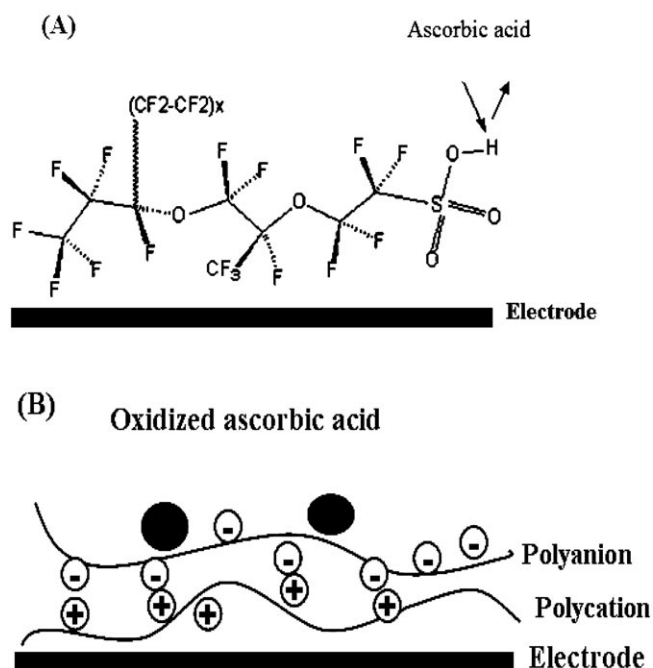


Fig. 6. Schematic diagram to illustrate the possible mechanism of Nafion (A) and polyion (B) matrix on the modified palladium electrode to eliminate interferences.

expected to be ca. 3%; whereas, with the polyion-modified electrode, the current change caused by the total ascorbic acid and uric acid is expected to be ca. 7.8%. This indicates that the present electrode can be used for accurate determination of glucose in sera. The higher interference level of the polyion membrane may be due to the lack of optimization of the synthetic process of the membrane.

4. Conclusions

A photo cross-linkable polymer was adapted to fabricate a planar electrode-based amperometric biosensor to achieve a multiuse or continuous analysis purpose. Nafion has been demonstrated to be effective to eliminate the interference response of electrochemically active species (ascorbic acid and uric acid) on the enzyme electrode. Furthermore, it can restrict the oxidized anionic interferent to adhere on its surface, thereby the fouling of the electrode was avoided. Notably, the stability of the proposed PVA-SbQ/GOD planar electrode is superior to the most commercially available membrane-covered electrodes which has a useful life of about ten days only. Compared to the conventional three-dimensional electrodes the proposed planar electrode exhibits a similar long-term stability, but is smaller, more responsive and more versatile. The manufacturing processes used in the semiconductor industry can be adapted to produce these electrodes at a unit cost that is low enough to ensure cost-effectiveness.

Table 1. The electrochemical response of Nafion and polyion modified enzyme electrodes to 5 mM glucose, ascorbic acid (AA) and uric acid (UA). Data are mean values of three measurements. Numbers in parentheses are the interference levels. The interference level is calculated as the ratio of electrochemical response of enzyme electrode to 5 mM AA or UA to the electrochemical response to 5 mM glucose.

Response (nA)	Glucose	AA	UA
Bare electrode [a]	159	227 (1.43)	202 (1.27)
Polyion modified electrode	153	140 (0.92)	100 (0.65)
Nafion modified electrode	162	39 (0.24)	34 (0.22)

[a] Containing 25% PVA-SbQ

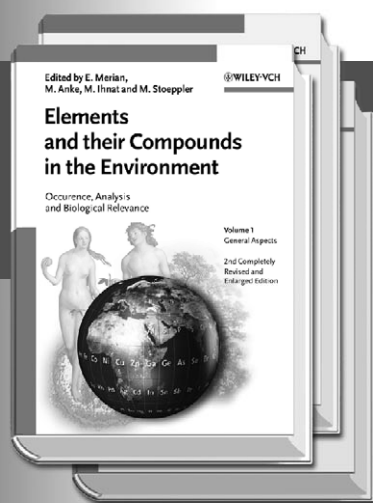
5. Acknowledgements

This work was financially supported by the National Science Council of the Republic of China, Taiwan under the Project No. NSC 93-2113-M-264-001 (KSC) and the Center for Nano Science and Technology of University System of Taiwan and the Ministry of Education of R. O.C. (Taiwan) (CJY).

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