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Simple and Effective Procedure for Immobilization of Oxidases onto MnO_2-bulk-modified, Screen-printed Carbon Electrodes

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ABSTRACT

Nafion® film entrapment of oxidases onto the surface of MnO_2-(5%, m:m) bulk-modified, screen-printed carbon electrodes was studied taking the inexpensive glucose oxidase as a model enzyme. The Nafion®-enzyme film was formed by drop-coating the suspension onto the surface of the electrode. Comparisons were made among biosensors developed by using as-received, neutralized, and diluted (to 1% in methanol, ethanol or phosphate buffer) Nafion® in a flow injection (FI) mode. The amperometric response to injections of standard glucose solution was the highest for biosensors produced using neutralized Nafion®. The effect of enzyme load per electrode (10–1190 μg) was investigated and 50 μg enzyme in the film per electrode was found to produce very good sensitivity as well as to be economical. Good reproducibility was achieved for three different biosensors (RSD of the means = 5.7%). The operational parameters for the glucose biosensor produced with this immobilization procedure were assessed and figures of merit obtained at the selected parameters. It has been observed that the effect of Nafion® to repel interfering ions like ascorbate and urate was lacking in this system. A preliminary investigation incorporating a hydrogen peroxide permselective membrane layer prior to enzyme immobilization gave promising result with polyurethane.

KEYWORDS

Amperometric glucose biosensor, flow injection analysis, Nafion® film entrapment, immobilization, screen-printed electrodes, MnO_2-modified electrodes.

1. Introduction

Solid electrodes based on heterogeneous carbon materials are used more frequently as electrochemical sensors and biosensors because of their availability in a variety of forms, low cost, broad exploitable potential window, low background current, chemical inertness, ease of chemical derivatization and modification, and suitability for various applications.[1,2] Various carbon-based electrodes have been designed from glassy carbon, reticulated vitreous carbon, graphite epoxy composites, pyrolytic graphite, carbon pastes, carbon inks and carbon fibres. Among these materials, carbon paste electrodes (CPEs) and screen-printed carbon electrodes (SPCEs) have gained widespread popularity in the development of electrochemical sensors and biosensors owing to their ease of preparation and modification, ease of surface renewal and reproducibility in case of CPEs, and mass-production of highly reproducible electrodes for SPCEs.[2–4]

In electroanalysis employing amperometric techniques there is an inherent problem of the dependence of the electrochemical reaction on the type of electrode. Many electroactive species undergo redox reactions at a slower rate than expected, requiring application of a higher potential. But at this over-potential, other species present in the sample may interfere, affecting the analytical reaction significantly. Sometimes, thermodynamically possible redox reactions may not be observed due to electrode fouling caused by adsorption or by chemical reactions involving the electrode surface. These phenomena can be controlled by deliberately attaching chemical reagents to the electrode surface so that manipulating the nature of the surface is possible and hence the name *chemically modified electrodes* was coined.[5–8] Heterogeneous carbon electrodes were among the first to be chemically modified.

The advantages of modified electrodes are reducing the overvoltage and hence the interference from other species, promoting electron transfer reactions that may increase the selectivity, specificity and reproducibility of the electrode surface, and improving the detection limit.[1–8]

Modified electrodes can be prepared by chemisorption, covalent bond formation between specific functional group on the electrode surface and the reagent, coating the electrode with polymeric films (including electropolymerization of monomers), and forming heterogeneous layers (e.g. thorough mixing of the modifier with carbon paste or carbon ink).[1,2] Among these, the simplest, most reliable and frequently used modification technique is admixing the modifier with conducting pastes and inks prior to putting the electrode in the desired geometry. In this context, numerous transition metal species (oxides and complexes) have been used as modifiers. Ferrocene derivatives, organometallics like ruthenium and osmium complexes, hexacyanoferrate, Methylene Blue and Methyl Viologen are most often employed.[8] A very recent example is the use of manganese dioxide. MnO_2 has been employed to modify CPEs and SPCEs in the production of sensors and biosensors for different analytes. Thus, the development and characterization of MnO_2 film/bulk-modified carbon paste and screen-printed amperometric sensors for H_2O_2,[9–12] uric acid[13] and ascorbic acid[14] have been successfully achieved. Moreover, glucose biosensors have been designed based on CPEs bulk-modified with MnO_2 and glucose oxidase (GOx)[15,16] as well as based on screen-printed

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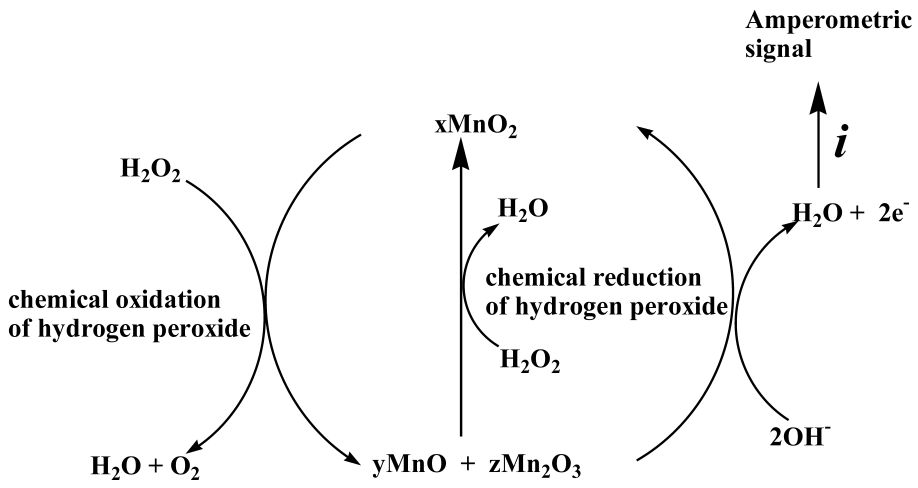

Scheme 1
Catalytic redox cycle of manganese dioxide and chemical oxidation of H_2O_2.[16,17]

amperometric sensors.[17,18] At an operational potential of 400–500 mV *vs* Ag/AgCl heterogeneous carbon electrodes bulk-modified with 3.8–5% MnO_2 responded to H_2O_2 (either directly present in the sample or as a product of the enzymatic oxidation). The tetravalent manganese is reduced to lower oxidation states by hydrogen peroxide and is reoxidized again electrochemically (Scheme 1).

In the aforementioned works that deal with biosensors based on MnO_2-modified electrodes, the biological recognition element has been introduced in the bulk of the electrodes. However, introducing expensive enzymes such as L-glutamate oxidase and L-amino acid oxidase in the bulk is not economically feasible since relatively large quantities of these enzymes are required. To alleviate this problem, Nafion® film entrapment of oxidases onto the surface of MnO_2- (5%, m:m) bulk-modified SPCEs was studied using the inexpensive glucose oxidase as a model enzyme.

2. Experimental

2.1. Materials

Glucose oxidase, GOx (EC 1.1.3.4 from *Aspergillus niger,* 210 U/mg solid) and D-glucose, anhydrous, were obtained from Sigma Chemicals Co., (St. Louis, MO, USA). Nafion®, perfluorinated ion-exchange resin, 5% (w/w) solution in lower aliphatic alcohols and water was from Aldrich (Aldrich-Chemie GmbH & Co KG, Steinheim, Germany). Polyurethane was synthesized according to Yun *et al.*[19] from poly-(tetramethylene ether glycol) and 4,4′-methylenebis(phenyl isocyanate). All other chemicals used were analytical reagent grade.

2.2. Reagents and Solutions

The water used was double-distilled in a quartz still and deionized with an ion exchange System (Nanopure, Barnstead). Phosphate buffer (0.1 mol L^{-1}) was prepared by mixing aqueous solutions (0.1 mol L^{-1}) of sodium di-hydrogen phosphate (Fluka) and di-sodium hydrogen phosphate (Fluka) to produce solutions of the required pH. A stock solution of glucose (5000 mg L^{-1}) was prepared by dissolving 0.5 g D (+) glucose in 100 mL of the corresponding working buffer solution, kept at room temperature overnight to facilitate α- β-mutarotation and stored at 4°C when not in use. Solutions of lower concentrations were prepared immediately before use. Hydrogen peroxide (p..a. Merck) was standardized by iodometric titration.[20] A stock solution containing 5000 mg L^{-1} was prepared freshly each day in the working buffer solution and stored at 4°C when not in use. Solutions of lower concentrations were prepared immediately before use. By the same token, stock solutions (4395 and 5000 mg L^{-1} respectively) of uric acid (sodium salt) and ascorbic acid were prepared by dissolving 0.5 g of each in 100 mL of the corresponding working buffer solution. Solutions of lower concentrations were prepared immediately before use.

2.3. Electrode Preparation

The same electrode preparation protocol reported elsewhere was employed.[21]

2.4. Enzyme Immobilization

Nafion® (5% solution) was diluted to 1% either in phosphate buffer (0.1 mol L^{-1}, pH 7.0), absolute ethanol, or absolute methanol. It was also neutralized by ammonia solution or used as-received. Adding a drop of ammonia solution to 1.5 mL Nafion® solution effected the neutralization and the pH adjustment was monitored by using litmus paper (Macherey-Nagel, Dueren, Germany). A weighed amount (in mg) of GOx was dissolved in phosphate buffer (0.1 mol L^{-1}, pH 7.0) and mixed with an equal amount (as the phosphate buffer) of Nafion® (diluted, neutralized or as-received) solution. Five μL (unless otherwise specified) of the resulting mixture were directly applied onto the active area of the SPCE (MnO_2-bulk-modified) surface (~0.40 cm^2 area), air-dried, another 5 μL aliquot applied, air-dried and inserted in the FIA system. The final enzyme load onto the electrode was estimated from the starting amount, assuming complete dissolution. No attempt was made to measure the enzyme activity retained after immobilization. The final concentration of Nafion® applied to the electrode was 0.5% in all diluted cases but 2.5% in the neutralized and as-received applications.

2.5. Flow Injection System

The flow injection system employed was basically the same as reported earlier.[21]

2.6. Preparation of Permselective Membranes

Cellulose acetate membranes were produced by spreading 2 mL of cellulose acetate (0.5% m:m) in cyclohexanone over water in a Petri dish (~9 cm in diameter). After evaporation of the cyclohexanone overnight, the membrane formed on the surface of the water was carefully transferred to the electrode surface.

In the case of drop-coated cellulose acetate, a solution (5%,

m:m) was prepared in a 1:1 mixture of acetone and cyclohexanone. Ten μL of this solution was drop-coated onto the surface of SPCE and air-dried. Polyurethane solutions (2, 5 and 10%) were prepared in a mixture of tetrahydrofuran and dimethylformamide (98 + 2, V + V). The SPCE was dip-coated (except the electric contact) with this solution and allowed to air-dry. Similarly, an aqueous solution containing hexaethyl cellulose (3.5%, m:m), polyethylene glycol (3%, m:m) and Triton® X-100 (0.02%, m:m) was prepared and the SPCE dip-coated with this solution.

3. Results and Discussion

3.1. Enzyme Immobilization

Nafion® film immobilization of oxidases has been performed with different Nafion® solutions and different electrode systems. Karayakin *et al.* used Nafion® solution diluted to 1% in ethanol and neutralized it with concentrated alkali hydroxides or concentrated ammonium hydroxide and then drop-coated the enzyme/electrolyte mixture onto a Prussian Blue-modified glassy carbon electrode.[22–25] Nafion® solution diluted to 0.5% in distilled water has also been used to immobilize GOx onto a platinum disk electrode[26] and onto a 1,1′-dimethyl-ferricinium-modified glassy carbon electrode.[27] Rishpon *et al.* used a Nafion® solution diluted to 0.5% in phosphate buffer (0.1 mol L^{-1}, pH 7.0) to immobilize GOx onto a platinum electrode[28] whereas Fortier *et al.* immobilized GOx onto a platinum disk electrode using a Nafion® solution diluted to 0.3% in methanol.[29] There is also a report where ammonium carbonate neutralization of Nafion® was performed followed by dilution to 1% in ethanol to immobilize GOx onto a Methylene Blue-modified glassy carbon electrode.[30] O'Halloran *et al.* immobilized GOx onto a Prussian Blue-bulk-modified SPCE using Nafion® neutralized by sodium carbonate and diluted to 0.25% before enzyme casting.[31] To our knowledge, there is no report on the comparison of Nafion® film immobilization effects in different solutions especially referring to the immobilization onto MnO_2-bulk-modified SPCEs.

3.1.1. Comparison of Different Nafion® Solutions

In this work, GOx was immobilized onto MnO_2-bulk-modified SPCE using Nafion® solution diluted to 1% either in phosphate buffer (0.1 mol L^{-1}, pH 7.0), ethanol or methanol. Enzyme electrodes were also prepared by using Nafion® (5%) either neutralized with $NH_3.H_2O$ or as-received. The total amount of enzyme in each case was 95.2 μg per electrode. Responses of such electrodes to injections of 100 μL of different glucose standard solutions with flow injection and amperometric detection is shown in Fig. 1.

Higher response was observed for the electrode prepared with neutralized Nafion® followed by the one produced with as-received Nafion®. The response was practically the same for enzyme electrodes prepared with diluted Nafion®, especially in the lower concentration ranges (20–60 mg L^{-1}) regardless of the solvent used for dilution. The lower response for the electrodes produced with diluted Nafion® compared to those produced with neutralized or as-received Nafion® might be due to leaching out of the enzyme from the less cross-linked polymer matrix. According to Rishpon *et al.* the Nafion® film has buffering effects on fluctuating oxygen concentrations due to higher solubility and lower diffusivity of oxygen in Nafion® than in solution.[28] Thus, in diluted Nafion®, this effect might diminish which might in turn contribute to the lower response compared to those obtained with undiluted solutions. The higher response in the electrode prepared with neutralized Nafion® compared to the one with as-received can be attributed to the lower pH of the latter affecting the enzyme activity. As-received Nafion® has a pH less than one[24,30] which possibly inactivates the enzyme during immobilization. The higher response with neutralized Nafion® is in agreement with a recent recommendation made by Karayakin *et al.* to use $NH_3.H_2O$ neutralization.[22]

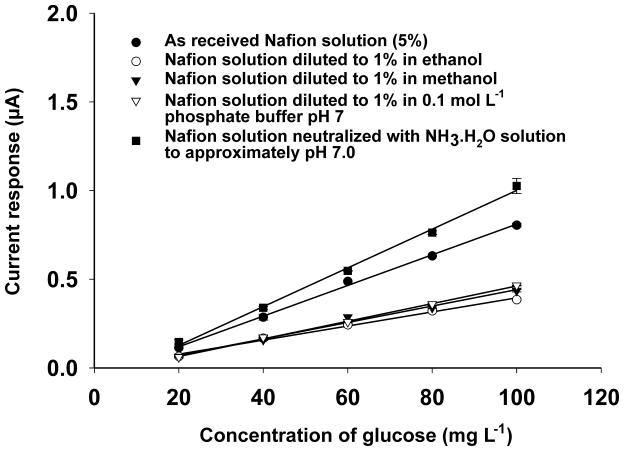

Figure 1 Comparison of amperometric responses of biosensors produced by enzyme castings using diluted (to 1% in different solvents), neutralized and as-received (5%) Nafion® solutions. The final enzyme load in each case was 95.2 μg in two subsequent layers. Working conditions: applied potential of 400 mV *vs* Ag/AgCl (3 mol L^{-1} KCl) reference electrode, flow rate 0.2 mL min^{-1}, injection volume 100 μL, carrier phosphate buffer (0.1 mol L^{-1}, pH 7.5). Measurements were done in triplicate.

Since drop-coating with casting (enzyme-polyelectrolyte) solutions results in random orientation of enzyme molecules after immobilization, a higher enzyme load is generally expected to give lower response because of molecular overcrowding that inhibits the substrate molecule to reach to the active centre of the enzyme and the intermediates to access to the transducer surface.[32]

To test the validity of this explanation with respect to the immobilization protocol discussed above, enzyme electrodes were prepared using Nafion® solutions (5%) either diluted with phosphate buffer (to 1%) or as-received with higher enzyme load than in the previous experiment (1000 μg/surface). Again the amperometric responses of these electrodes were monitored by injecting 100 μL of different glucose standard solutions.

Still higher response was observed for the biosensor prepared by using as-received Nafion® solution (data not shown). When using the diluted solution, the final concentration of Nafion® in the casting mixture was 0.5% (see Experimental) where as in the other case it was 2.5%. Thus, the lower the concentration of Nafion® the higher the rate of leaching out of the enzyme and *vice versa.* But the difference in response was not that much pronounced as in the case with lower enzyme load indicating a certain degree of molecular overcrowding in using as-received Nafion® solution.

3.1.2. Optimum Enzyme Load

Figure 2 shows the response of biosensors with different amounts of GOx immobilized in the Nafion® films to injections of 100 μL standard glucose solution (100 mg L^{-1}). A sharp increase was observed up to around 50 μg enzyme in the film (~0.50 cm^2 electrode area) and then slight increase up to 720 μg followed by leveling off. At higher enzyme loads cracking of the enzyme and carbon ink layers was seen. Thus, for economic reason (since the aim was to characterize this immobilization procedure for expensive enzymes) and to avoid risk of damaging, the recognition as

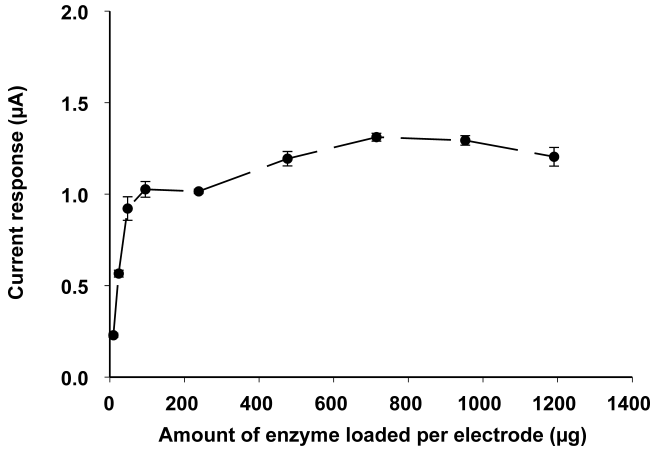

Figure 2 Optimization of enzyme load applied in two layers in neutralized Nafion® solutions (~5%). Working conditions: applied potential of 400 mV *vs* Ag/AgCl reference electrode, flow rate 0.2 mL min^{-1}, concentration of standard glucose solution 100 mg L^{-1}, injection volume 100 μL, and carrier phosphate buffer (0.1 mol L^{-1}, pH 7.5). Measurements were done in triplicate.

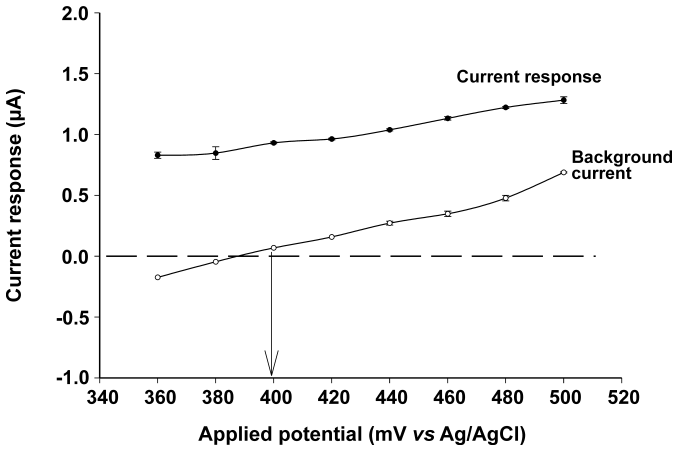

Figure 3 Dependence of the current response of glucose on the applied potential. Enzyme load 47.6 μg in two subsequent layers (neutralized Nafion® solution). Working conditions: flow rate 0.2 mL min^{-1}, concentration of standard glucose solution 100 mg L^{-1}, injection volume 100 μL, carrier phosphate buffer (0.1 mol L^{-1}, pH 7.5). Measurements were done in triplicate.

well as the conductive layer 47.6 μg was used in subsequent experiments.

3.1.3. Reproducibility of Biosensor Production

One of the major problems associated with the drop-coating technique is a lack of reproducibility.[32] Comparison was made among three biosensors produced using the same batch of SPCEs but individual preparation of casting solutions. With an enzyme load of 47.6 μg in two subsequent layers and the working conditions mentioned in Fig. 2, the relative standard deviations of the different batches of measurements were between 3 and 6% (three measurements for each batch), whereas the mean values of the batches showed a relative standard deviation of 5.7%. The deviations are within acceptable limits and are due to experimental errors during preparation and measurement.

3.2. Optimization of Operational Parameters

3.2.1. Applied Potential

The concentration of the modifier MnO_2 used was the same as in previous reports.[9–18] Schachl *et al.* demonstrated that MnO_2-bulk-modified electrodes show highest response in the potential range 400 to 500 mV *vs* Ag/AgCl.[12,18] Figure 3 shows the dependence of the current response and the background current on the applied potential. At 400 mV *vs* Ag/AgCl the background current is nearly zero and the current response is higher than at lower potentials.

At potentials below 400 mV, decreasing responses were observed and the background current was reductive which leads to a gradual leaching of the modifier due to formation of soluble Mn(II) species. At potentials higher than 400 mV, even though an increasing response was obtained the background current was also increasing, affecting the reproducibility of the measurements. Thus, a working potential of 400 mV was chosen for subsequent measurements.

3.2.2. Flow Rate

Like any measurements in flow injection analysis, the amperometric current response was affected by a change in the flow rate (Fig. 4). The current response exhibited an inverse relationship with the flow rate, i.e. the higher the flow rate the lower the current response. In the range of flow rate studied, the background current increased with flow rates, which is in agreement with previous reports.[9–11]

At higher flow rates the residence time of the analyte in close proximity to the biological recognition element (the enzyme) was very small and dispersion was higher, hence lower peak height. With a constant concentration of analyte in the carrier one would expect an increase of the signal with the flow rate (proportional to the cube root of the flow rate for a thin-layer cell). As the sample is injected, only a transient signal is obtained which is determined by the dispersion (increasing with higher flow rates) and the kinetics of the enzymatic reaction. Highest signal was obtained at 0.1 mL min^{-1} within the investigated range but due to the long relaxation time required and hence the slow sample throughput, 0.2 mL min^{-1} was chosen as working flow rate.

3.3. Figures of Merit

Figure 5 shows typical responses of the biosensor upon injections of 100 μL of different concentrations of standard glucose solutions. Employing the optimum parameters discussed above,

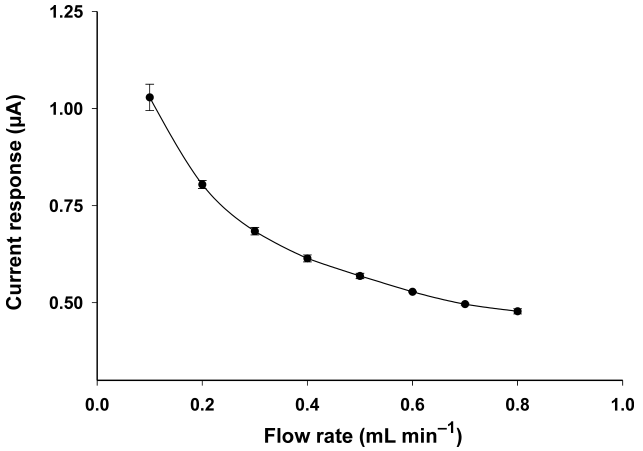

Figure 4 Dependence of the current response of glucose on the flow rate. Enzyme load 47.6 μg in two subsequent layers (neutralized Nafion® solution). Working conditions: applied potential of 400 mV *vs* Ag/AgCl, concentration of standard glucose solution 100 mg L^{-1}, injection volume 100 μL, carrier phosphate buffer (0.1 mol L^{-1}, pH 7.5). Measurements were done in triplicate.

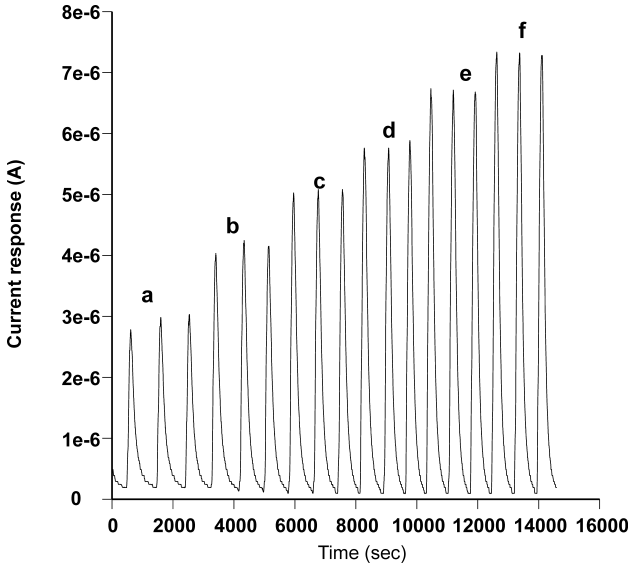

Figure 5 Typical response of the glucose biosensor developed for concentrations of a) 150, b) 200, c) 250, d) 300, e) 400 and f) 500 mg L^{-1} glucose at an applied potential of 400 mV *vs* Ag/AgCl, flow rate of 0.2 mL min^{-1}, carrier phosphate buffer (0.1 mol L^{-1}, pH 7.5).

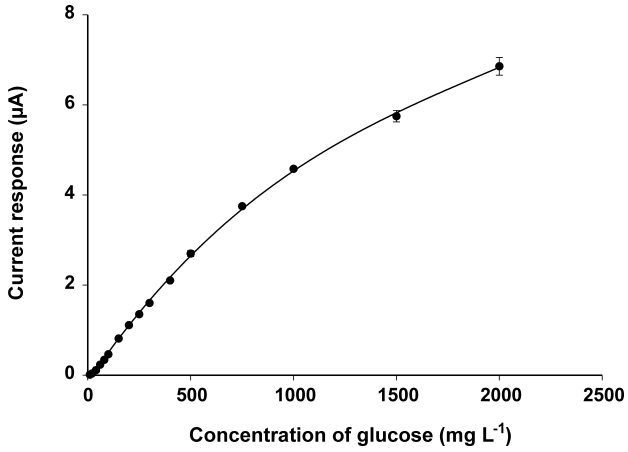

Figure 6 Calibration curve for different concentrations of glucose (n = 3). Working conditions: applied potential of 400 mV *vs* Ag/AgCl, flow rate 0.2 mL min^{-1}, injection volume 100 μL, carrier phosphate buffer (0.1 mol L^{-1}, pH 7.5).

the biosensor produced by Nafion® film immobilization of GOx was used to produce calibration curve in the concentration range 10–2000 mg L^{-1} (Fig. 6). Though there is linear relationship between concentration and current response in the lower concentrations the entire curve can best be expressed by a non-linear regression (i [μA] = $-0.072 + 5.92 \times 10^{-3}$ c[mg L^{-1}] – 1.26×10^{-6} c^2 [mg^{-2} L^{-2}], r^2 = 0.999). The detection limit (as 3σ values) from five injections of 100 μL standard glucose solution (10 mg L^{-1}) was found to be 3.7 mg mL^{-1}. A relative standard deviation of 3.1% was recorded for six injections of 20 mg L^{-1} standard glucose solution.

3.4. Interference Elimination

It has been previously reported that the MnO_2-bulk-modified SPCE suffers from interfering substances such as ascorbate and urate, and even this property was exploited to produce sensors for those interfering compounds.[9–18] Despite the lower applied potential and expected effect of the Nafion® film which is known to repel anionic species such as urates and ascorbates, the biosensor developed in this work was also prone to interference from these compounds. The failure of the Nafion® layer to repel the anionic species could be due to a compensation of the negative charge of the sulfonic acid groups by rather tightly bound ammonium ions after neutralization. Thus, the film itself does not act as a charge barrier anymore. Physiological concentration of ascorbic acid in healthy adult (10^{-4} M)[33] gave a current equivalent to that of 100 mg L^{-1} glucose solution. Thus, attempts were made to produce H_2O_2 permselective membranes onto the active surface of SPCE prior to immobilization of the enzyme.

Cellulose acetate is a commonly used membrane for permselectivity of hydrogen peroxide while retaining ascorbic and uric acids. The cellulose acetate membrane was prepared according to Yamamoto *et al.*[34] but transferring it onto the surface of SPCE was not successful because of tearing out of the very thin membrane. To circumvent this problem the procedure was modified as follows. Fifty μL of water was floated on the flat surface of the SPCE onto which 5 μL of 5% cellulose acetate (CA) in a 1:1 mixture of acetone (AC) and cyclohexanone (CH) was pipetted. After 20 minutes the SPCE was tilted to decant the water so that the cured membrane attaches itself to the electrode surface.

Though membrane preparation was successful, it was damaged by the Nafion® solution during immobilization. Thus, dip- and drop-coating were again attempted to form cellulose acetate layer. Direct application of cellulose acetate solution disrupted the integrity of the carbon electrode because of the organic solvents used to dissolve cellulose acetate as noted by Gilmartin and Hart.[35] Polyurethane also exhibits some permselectivity to small molecules and retards larger ones.[36] To this effect 5% polyurethane in 98% tetrahydrofuran and 2% dimethylformamide was dip-coated onto the SPCE. Ge *et al.*[37] used hydroxyethyl cellulose-polyethylene glycol-Triton® X-100 layer as protective membrane and the effect of such a layer was also tested in this work. Table 1 shows interference elimination effect of these

Table 1 Interference eliminating effect of different membranes drop- or dip-coated onto the surface of screen-printed electrodes prior to enzyme immobilization. Measurements were done in triplicate.

Membrane		Current response [nA] to 250 μL injection of:			
Type	Description	15 mg L^{-1} ascorbic acid [a]	50 mg L^{-1} ascorbic acid	70 mg L^{-1} uric acid [b]	50 mg L^{-1} H_2O_2
A	Drop-coated 5% CA in AC:CH (1:1)	11.2 ± 2.2	41.3 ± 3.4	46.6 ± 2.4	180 ± 11
B	Dip-coated 5% PU in 98% THF and 2% DMF	No current detected	16.6 ± 1.7	17.5 ± 2.6	212 ± 11
C	Dip-coated 3.5% HEC, 0.02% Triton X-100, 3% PEG	1648 ± 13	N/A	4460 ± 310	2300 ± 230

CA: cellulose acetate; AC: acetone; CH: cyclohexanone; PU: polyurethane; THF: tetrahydrofuran; DMF: N, N-dimethylformamide; HEC: hydroxyethyl cellulose; PEG: polyethylene glycol

[a] Plasma concentration for healthy adult ranges from 4 to 15 mg L^{-1} [ref. 31].

[b] Serum concentration for healthy adult ranges from 23 to 76 mg L^{-1} [ref. 31].

Table 2 Comparison of amperometric FI response to injections of 250 μL standard solution of potential interferants alone and mixed with standard glucose solution (100 mg L^{-1}). Measurements were done in triplicate.

Analyte injected	Current response [nA], $n = 3$
50 mg L^{-1} ascorbic acid	420 ± 22
50 mg L^{-1} ascorbic acid + 100 mg L^{-1} glucose	680 ± 19
70 mg L^{-1} uric acid	376 ± 04
70 mg L^{-1} uric acid + 100 mg L^{-1} glucose	599 ± 12
100 mg L^{-1} glucose	243 ± 01

membranes formed by drop- or dip-coating directly on the surface of SPCEs, prior to enzyme immobilization.

The polyurethane membrane gave promising result of permselectivity. No current was detected for the physiological concentration of ascorbic acid and very small (17 nA) for physiological concentration of uric acid. Compared to the less efficient membrane prepared according to Ge *et al.*,[37] the polyurethane layer reduced interference of uric acid by 99.6% and of ascorbic acid by 100%.

Prompted by this promising preliminary result, Nafion® film immobilization of GOx was performed onto polyurethane coated MnO_2-bulk-modified SPCE. There was no response detected for injections of 250 μL standard glucose solution (100 mg L^{-1}) when 10, 47.6, or 95.2 μg GOx was used for immobilization per electrode. Therefore, the concentration of polyurethane was reduced from 5 to 2% and the enzyme load increased to 238 μg per electrode.

Table 2 presents the interference elimination effect of the polyurethane membrane on the glucose biosensor. The different result compared to Table 1 can be explained by the fact that a lower amount of polymer used here (2% acompared to 5% previously). Moreover, the polymer layer might have been damaged by high concentration of hydrogen peroxide produced from enzymatic oxidation of glucose (up to 2000 mg L^{-1} glucose solution was encountered by this enzyme electrode).[38]

The result obtained by incorporating permselective membranes onto MnO_2-modified electrode system prior to immobilization is highly promising and requires further research.

4. Conclusions

This work clearly demonstrated the effectiveness of Nafion® film immobilization of oxidases on MnO_2-bulk-modified SPCEs. It has been shown that the immobilization technique is highly economical, fast, reproducible and simple. The glucose biosensor developed with this immobilization method exhibited similar characteristics to the one produced by bulk-modification of the carbon ink with the enzyme. A permselective polyurethane membrane applied onto the SPCE prior to immobilization gave promising results in eliminating interferences from the most notorious interfering species in biosensor research, namely ascorbate and urate. In continuous-flow mode (FIA) the polyurethane membrane could be damaged by repetitive exposure to hydrogen peroxide produced from the enzymatic reaction. However, it can effectively be used to retain the interferents from reaching to the active electrode surface in single-shot biosensors.

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