Anal. Chem. **2002,** *74,* 1597−1603

Optimal Environment for Glucose Oxidase in Perfluorosulfonated Ionomer Membranes: Improvement of First-Generation Biosensors

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An optimal environment for glucose oxidase (GOx) in Nafion membranes is achieved using an advanced immobilization protocol based on a nonaqueous immobilization route. Exposure of glucose oxidase to water−organic mixtures with a high (85−95%) content of the organic solvent resulted in stabilization of the enzyme by a membrane-forming polyelectrolyte. Such an optimal environment leads to the highest enzyme specific activity in the resulting membrane, as desired for optimal use of the expensive oxidases. Casting solution containing glucose oxidase and Nafion is completely stable over 5 days in a refrigerator, providing almost absolute reproducibility of GOx−Nafion membranes. A glucose biosensor was prepared by casting the GOx−Nafion membranes over Prussian Blue-modified glassy carbon disk electrodes. The biosensor operated in the FIA mode allows the detection of glucose down to the 0.1 μM level, along with high sensitivity (0.05 A M^{-1} cm^{-2}), which is only 10 times lower than the sensitivity of the hydrogen peroxide transducer used. A comparison with the recently reported enzyme electrodes based on similar H_2O_2 transducers (transition metal hexacyanoferrates) shows that the proposed approach displays a dramatic (100-fold) improvement in sensitivity of the resulting biosensor. Combined with the attractive performance of a Prussian Blue-based hydrogen peroxide transducer, the proposed immobilization protocol provides a superior performance for first-generation glucose biosensors in term of sensitivity and detection limits.

The development of enzyme electrodes has remained a prime focus of many researchers since the pioneering studies of Clark and Lyons in 1962[1] or by Updike and Hicks in 1967.[2] Immobilization is the key to the development of enzyme-based biosensors. The importance of the immobilization procedure for biosensor development is now outlined in IUPAC recommendations, stating that the only devices containing a "biological recognition element which is retained in direct spatial contact with an electrochemical transduction element" are considered as biosensors.[3]

Among the various enzyme immobilization protocols, an entrapment in polymer membranes is a general one for a variety of transducers. Formation of the membrane from polymer solution in organic solvent on any surface is more simple and reproducible compared to chemical polymerization. We believe that the simplicity of this immobilization procedure would provide the reproducibility of the resulting biosensors strongly required for their mass production.

Polyelectrolyte Nafion has found wide use for development of the enzyme-containing membranes. Among its main advantages is that Nafion provides a biocompatible interface with mammalian tissue and hence offers the potential for use with implantable sensors.[4] The method for membrane formation is a simple dipping of the electrode into the polyelectrolyte solution or casting a small volume of the solution onto the electrode surface and allowing the solvent to evaporate. The resulting membranes possess a high adhesion to the surface and a low swelling in aqueous media.

In early studies, the formation of enzyme−Nafion membranes was carried out from Nafion solutions excessively diluted with water in order to prevent denaturation of protein with organic solvent.[5,6] However, Nafion membranes deposited from such water−organic mixtures with low content of organic solvent seemed to be nonuniform, which was later confirmed through the study of ion diffusion.[7]

The investigation of the enzymes in water-miscible organic solvents trivially called "nonaqueous enzymology" about 12 years ago became an independent part of modern biochemistry and enzymology.[8−10] In concentrated organic solvents with the water

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10.1021/ac0155409 CCC: $22.00
Published on Web 03/05/2002

content less than 10−15%, the enzymes are rather stable and even retain their activity.[9,11] Recent studies even demonstrated improvement of the enzyme activity in concentrated organic solvents.[12]

The methodology of our approach is, however, quite different from "nonaqueous enzymology". For immobilization, we propose to entrap the enzymes in water-insoluble polymers. To improve the properties of the resulting membranes, we propose to deposit them from real solutions of polymers. Thus, during immobilization, the enzymes have to be exposed to organic solvents. However, the activity of the immobilized enzymes is required only in aqueous solutions in which the resulting biosensors are operated. For this approach, it is only important that the enzymes are able to retain their catalytic properties after exposure to organic solvents.

We already reported that after exposure in optimal conditions to concentrated organic solvents with water content of 5−15%, the enzymes retain 100% of their initial activity.[13] Moreover, we observed the improved activity of some of the enzyme samples after this procedure,[13] which was a precursor of the later achievements on enzyme activation in organic media.[12] The high remaining activity of the enzymes after their exposure to organic media allows us to carry out the immobilization in Nafion membranes without excessive dilution of the polyelectrolyte solution with water. Electrochemical biosensors made accordingly displayed both improved response and enhanced stability.[13,14] However, the immobilization protocol under discussion included exposure of the enzymes to pure organic solvents, which resulted in nonuniform suspensions. This dramatically affected the reproducibility of the resulting membranes. The biosensors made accordingly were not able to reach submicromolar detection limits despite the advantageous analytical performances of the hydrogen peroxide transducers used.

We believed we were able to improve the key properties of the enzyme−Nafion membranes by controlling the step of enzyme exposure to concentrated organic solvents. On the basis of the knowledge that different polyelectrolytes can stabilize proteins,[15] even in water−organic mixtures,[16] we decided to check the membrane-forming polyelectrolyte itself as a potential stabilizer. Finding the optimal environment for glucose oxidase in Nafion membranes provides a dramatic improvement of the corresponding biosensors concerning their sensitivity and detection limits and gives a methodology for optimal use of the expensive oxidases in bioanalytical systems.

EXPERIMENTAL SECTION

Materials. Experiments were carried out with MilliQ water from a Millipore MilliQ system. All inorganic salts were obtained at the highest purity and afterward were recrystallized from MilliQ water. Absolute ethanol was prepared by distillation of sodium alcoholate and used immediately. Nafion (5% solution in light alcohols containing 15−20% H_2O) was purchased from Aldrich (Steinheim, Germany). Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (type VII-S, 180 IU/mg) was produced by Sigma (St. Louis, MO). D-(+)-Glucose was purchased from ICN Biomedicals (Irvine, CA).

Instrumentation. The activity of glucose oxidase (GOx) was measured by O_2 consumption with a Clark-type oxygen sensor with platinum indicator and Ag|AgCl|1 M KCl reference electrodes separated with a Teflon membrane from the analyzing solution. Homemade polarograph and recorder (19-8004-01) (Pharmacia) were used with Clark-type sensor. The GOx−Nafion membrane was formed on the bottom of a 1.56-mL poly(methyl methacrylate) cell made for the Clark-type sensor. The cell was hermetically connected to the sensor and stirred during the experiments with magnetic stirrer. The cell was thermostated at 25 °C using a Haake thermostat (SK75).

Optical density of GOx suspensions in Nafion was measured with an LKB-Ultraspec UII (Bromma, Sweden) spectrophotometer.

The flow injection system consisted of a Cole Parmer (Vernon Hills, IL) peristaltic pump (7519−10), homemade flow-through wall-jet cell with 0.5-mm nozzle positioned at 1-mm distance from the surface of disk electrode, (Ag|AgCl|1 M KCl) reference, homemade injector, and Metrohm potentiostat (641-VA) or Solartron electrochemical interface model 1286 interfaced to an IBM PC. Flow rates used were in the range 0.5÷1 mL min^{-1}. In FIA experiments, the peak current values were taken for data treatment, sample volume was 50 μL, and working electrode potential was 0.0 V, such potential allowing hydrogen peroxide reduction on Prussian Blue-modified glassy carbon disk electrodes.

Electrochemical deposition was carried out in a three-compartment electrochemical cell containing a platinum net auxiliary electrode and a Ag|AgCl reference electrode in 1 M KCl. The cell construction allowed deaeration of the working electrode space. Glassy carbon disk electrodes (diameter 1.5 mm) were used as working electrodes. Prior to use, the glassy carbon electrodes were mechanically polished with alumina powder (Al_2O_3, 1 μm) until a mirror finish was observed.

Glucose Oxidase−Nafion Membrane Preparation. The 1% Nafion stock solution was prepared by dilution of 5% Nafion with absolute ethanol. The 1% Nafion solution was neutralized by different alkalies (concentrated alcohol alkalies or concentrated ammonium hydroxide solution) up to pH 5.5, the latter being controlled with indicator paper Lachema. The resulting 1% Nafion solution (pH 5.5) was diluted with absolute ethanol to the final Nafion concentration.

GOx suspensions and various preparations of GOx−Nafion casting solutions in water−ethanol mixtures were placed into a 1-cm quartz cuvette (volume 3 mL). Optical densities were measured at 800 nm.

The casting solution containing glucose oxidase and Nafion in a water−ethanol mixture with a high (>85%) content of ethanol was prepared as follows. The lyophilized enzyme samples were dissolved in water to a final concentration of 20−60 mg mL^{-1}. It was shown that such concentrations caused no GOx inactivation. Finally, the casting solution was made by addition of a water−ethanol mixture with or without Nafion in different concentrations to the enzyme aqueous solution. All water−alcohol mixtures are given in volume percents.

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The GOx−Nafion membranes were formed on the bottom of a poly(methyl methacrylate) cell made for the Clark-type sensor (target surface area diameter 10 mm) or over Prussian Blue-modified electrodes by syringing of, respectively, 25 or 5 μL of GOx−Nafion casting solution to the target surface and allowing the solvent to evaporate. On the bottom of the Clark sensor cell, the membranes were dried for 1 h at room temperature and then washed with the phosphate buffer, pH 6.0, for 5 min with continuous stirring.

The resulting activity of the enzyme-containing membranes in glucose uptake was determined by the rate of oxygen consumption with a conventional Clark-type sensor in stirred 0.02 M acetate buffer, pH 5.5, containing 0.1 M KCl at 25 °C after injection of glucose to a final concentration of 0.05 M.

Stability of the Casting Solution and the Resulting Glucose Oxidase−Nafion Membranes. The shelf life of enzyme−Nafion membranes was tested as follows. After preparation on the bottom of the Clark-type cell, the membranes were kept in the dry state in a refrigerator (+4 °C) for up to two weeks. Then the cells were taken out one by one and the activity of the membranes was tested for the first time.

The stability (shelf life) of the casting solution containing glucose oxidase and Nafion in the water−organic mixture was tested similarly. The casting solution was kept in the refrigerator (+4 °C), and after a certain time it was used for preparation of the enzyme−Nafion membranes, which were tested immediately with the conventional Clark-type sensor.

Operational stability of GOx−Nafion membranes was investigated as follows. Freshly prepared membranes on the bottom of the Clark electrode cell were repeatedly tested several times. Prior to each measurement, the cells were thoroughly washed with water and dried.

Preparation and Testing of the Glucose-Sensitive Biosensor. Electrodeposition of Prussian Blue was done by applying a constant cathodic current of 80 μA cm^{-2} for 50 s. The initial solution contained 4 mM $K_3[Fe(CN)_6]$ and 4 mM $FeCl_3$. The supporting electrolyte was 0.1 M KCl with 0.1 M HCl. After deposition, the Prussian Blue films were activated in the same supporting electrolyte solution used for film growth, by cycling the applied potential in a range of −0.05 to +0.35 V at a sweep rate of 40 mV s^{-1} 10−15 times until a stable voltammogram was obtained. Then the electrodes were heated at 100 °C for 1 h and cycled ∼10 times in 0.02 M phosphate (pH 6.0) containing 0.1 M KCl (−0.05 to +0.35 V, 40 mV s^{-1}). The total amount of deposited Prussian Blue (∼6 nmol cm^{-2}) was estimated from cyclic voltammograms. GOx−Nafion membranes were deposited over Prussian Blue-modified electrodes as described above.

Flow injection glucose analysis was carried out in 0.05 M phosphate buffer, pH 6.0, with 0.1 M KCl at 0.0 V (Ag|AgCl|1 M KCl), which enables hydrogen peroxide reduction on the Prussian Blue-modified glassy carbon electrode. The flow rate was 0.5 mL min^{-1}. Sample volume was 50 μL.

RESULTS AND DISCUSSION

The homogeneity of an enzyme suspension in a water−organic mixture can be estimated by the dispersion of visible light. The optical density at 800 nm of a glucose oxidase suspension in concentrated organic solvent is dependent on the Nafion content in the initial water−ethanol mixture (Figure 1). The dependence

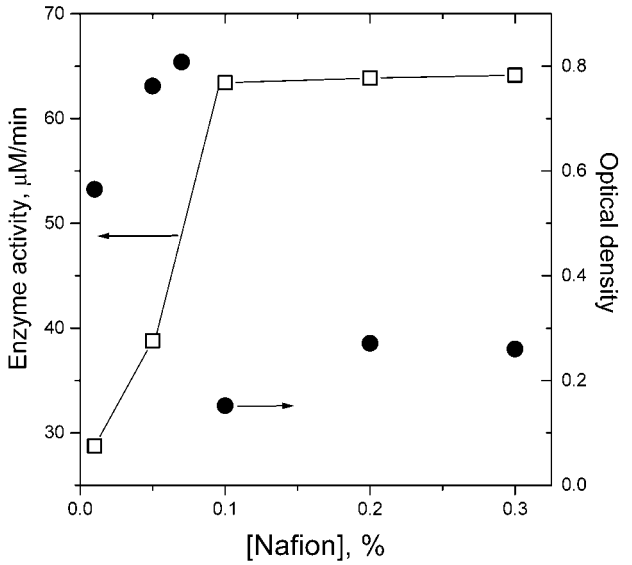

Figure 1. Effect of Nafion on the exposure of glucose oxidase to concentrated ethanol: (□) activity of the resulting membrane in glucose uptake; (●) 800-nm optical density of glucose oxidase−Nafion−ethanol mixture; 1.5 mg mL^{-1} glucose oxidase, 7.4% of water.

has two basic regions: high density at low Nafion concentrations and low density at high concentrations. The threshold concentration is between 0.07 and 0.1%. A decreased optical density obviously indicates a more homogeneous suspension, which is formed when the enzyme is suspended by the Nafion solution in water−ethanol mixtures with concentrations not lower than 0.1%.

The suspension procedure also affects the final activity of the enzyme-containing membranes in glucose uptake as also seen in Figure 1. In the course of this experiment, Nafion content in the casting solution remained the same (0.3%), whereas its concentration varied in the water−organic mixture used for enzyme suspension. Figure 1 shows that the membrane activity increased with Nafion concentration and reached a plateau at a Nafion content of 0.1%. Data at zero Nafion content are omitted due to their high distribution.

Based on the facts of the decreased optical density of the enzyme suspension and the improved membrane activity reached in a similar concentration region of Nafion, one can conclude that the membrane-forming polyelectrolyte is able to decrease both precipitation and denaturation of the enzyme upon its exposure to concentrated organic solvent. Polyelectrolyte can wrap up protein macromolecules or their conglomerates, making their solutions in concentrated organic solvents more stable.

The necessary step for immobilization of glucose oxidase in Nafion is a neutralization of polyelectrolyte[13,17] because of the high lability of this particular enzyme at extreme pH values.[18] It is known, however, that the substitution of protons by metal ions in Nafion membranes affects their microstructure.[19,20] The choice of cation for the neutralization step is probably also important for the immobilization procedure.

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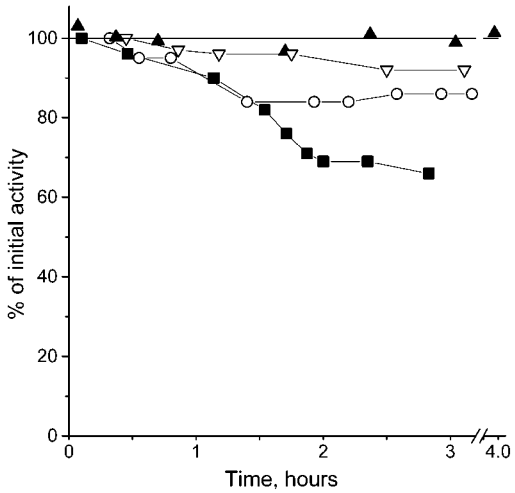

Figure 2. Operational stability of the glucose oxidase−Nafion membranes made with the use of different neutralizing alkalies: (■) KOH, (○) NaOH, (▽) LiOH, and (▲) NH_4OH.

Indeed, as seen in Figure 2 the operational stability of the enzyme-containing Nafion membranes is dependent on the metal ion used for neutralization and decreased in the following order: $NH_4^+ > Li^+ > Na^+ > K^+$. The metal ion radii are increased and the hydrated metal ion radii are decreased: $Li^+ < Na^+ < K^+$. However, this does not explain the 100% operational stability of the glucose oxidase−Nafion membranes reached with the use of NH_4^+ because the hydrated radii of potassium and ammonium ions are quite similar. A possible explanation for the unusual ammonium effect can be found from methods in biochemistry. Ammonium sulfate ($(NH_4)_2SO_4$) is known to be the best inorganic compound for precipitation of proteins from aqueous media which provides the highest degree of stabilization. Since polar substituents in Nafion are sulfo groups, after its neutralization with NH_4OH the ammonium sulfate residues in the polymer matrix are formed, which may stabilize the protein in the resulting membrane.

Achievement of the high stability of the glucose oxidase-containing Nafion membranes upon operation (Figure 2) was extremely important, and we decided to investigate how the main components of the casting solution affect it. Figure 3 displays a three-dimensional plot presenting a hyperplane as a border of 100% membrane stability during 3 h. Each point in the hyperplane is the maximum content of water in water−ethanol mixture at the given enzyme and Nafion concentrations, which allows the achievement of complete stability of the resulting membrane. A higher content of water in casting solution reduces the membrane stability.

Unexpected maximums in the hyperplane at an enzyme content of 1−1.5 mg mL^{-1} are observed (Figure 3). As the Nafion content is increased, a general shift of the abscissa maximum to higher enzyme concentrations is observed. Such extremes can be explained in terms of achievement of the optimal enzyme-to-Nafion ratios in the casting solution, which provide the operational stability of the resulting membranes.

The dependence of the specific activity of the immobilized glucose oxidase, assessed as the ratio of measured activity and mass of deposited GOx, with increase of the enzyme concentration in the casting solution is presented in Figure 4. Decrease of the specific activity with the increase in enzyme concentration is observed. For two curves of the five shown, a significant maximum of specific activity in the range of 1−1.5 mg mL^{-1} enzyme concentration is observed. In addition, when the Nafion concentration is 0.3%, an extreme of the specific activity shifted to lower enzyme concentrations seems to appear. The significance of the extremes is statistically clarified (see caption to Figure 4). The abscissa of the maximum is shifted to higher enzyme concentrations as the Nafion content in the casting solution is increased.

Observation of the extremes of both the membrane specific activity and its stability at similar enzyme concentrations and the enzyme-to-Nafion ratios points to the achievement of the optimal environment for the enzyme in the polyelectrolyte membrane. Moreover, since molar concentrations of the enzyme and the polyelectrolyte in the casting solution are of a similar order of magnitude, the observed extremes may indicate the formation of the enzyme−polyelectrolyte complex at a molecular recognition level in concentrated organic solvents. For practical application, finding of the optimal enzyme content in the casting solution, which provides the highest specific activity, is important for some expensive oxidases.

The operational stability of the glucose oxidase−Nafion membranes can be estimated from Figure 2. Despite the fact that during the measurements of the activity the enzyme−Nafion membrane was on the bottom of the cell, and a magnetic stirrer was in contact with the membrane, its activity was completely stable within first 4 h. The shelf life of the glucose oxidase−Nafion membranes is shown in Figure 5. No decrease of the membrane activity was observed within two weeks upon storage in a refrigerator.

Since some of the important enzymes (e.g., glutamate oxidase) are extremely expensive, the immobilization approaches must provide only minor use of the protein. For this reason, the entrapment into conducting polymers during electropolymerization,[21,22] which results in wasting of >90% of the enzyme sample, became less popular nowadays in the case of conventional electrodes. Procedures similar to the proposed one are more progressive. However, the possibility of storage of the enzyme−polyelectrolyte solution is highly attractive.

Figure 5 illustrates the stability of the casting solution of glucose oxidase and Nafion kept in a refrigerator. It is seen that the membranes made from the same solution during the first 5 days show the same activity in glucose uptake. Only after the seventh day was a decrease of the resulting membrane activity (∼10%) observed. Thus, one can use the same enzyme−polyelectrolyte solution for immobilization of the enzymes during the whole business week.

The reproducibility of the glucose oxidase−Nafion membranes is seen in Figure 5. When the same casting solution is used, the maximum deviation in membrane activity is ∼2%. This is, however, a precision limit for kinetic investigations (see Figure 2). Membranes prepared from different enzyme−polyelectrolyte solutions possess a higher deviation in their activity mainly due to the error in the weighing of small amounts of glucose oxidase.

As an application of the proposed immobilization protocol, the glucose-sensitive amperometric electrode was made. The trans-

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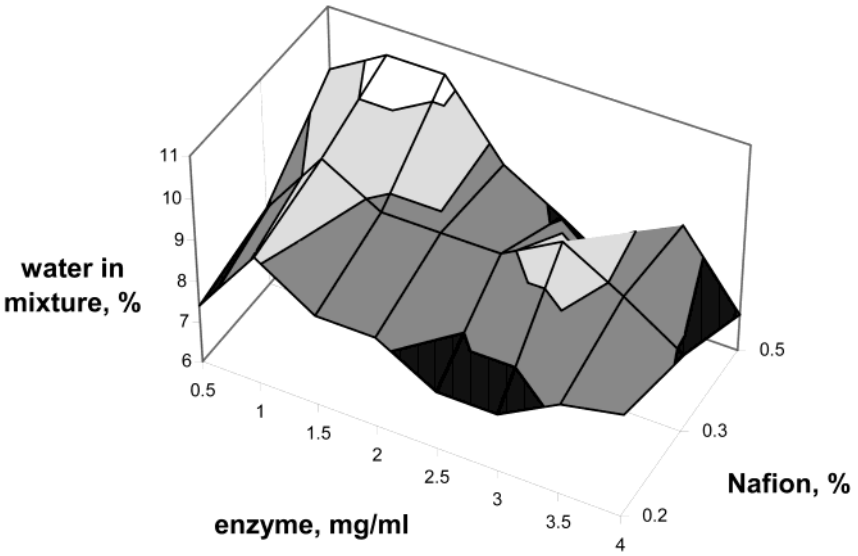

Figure 3. Hyperplane display of the maximum content of water in a water−ethanol mixture, which allows the achievement of 100% (3 h) stability of the resulting membrane.

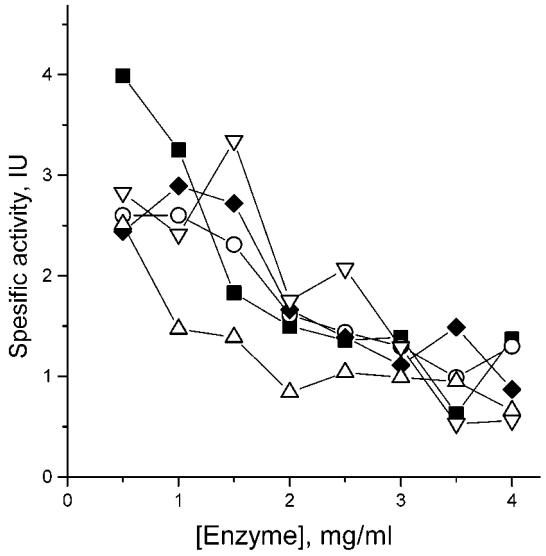

Figure 4. Specific activity of glucose oxidase−Nafion membranes (IU: μmol min^{-1} of the converted substrate per mg of GOx) as a function of both enzyme and polyelectrolyte concentration in the casting solution): (■) 0.2, (○) 0.3, (◆) 0.5, (▽) 0.8, ans (△) − 1.0% Nafion. Each point is the mean of not less than of 10 independent experiments. Standard deviation was ∼10−15%; error bars omitted for clarity of the plot indicate significance of the extremes.

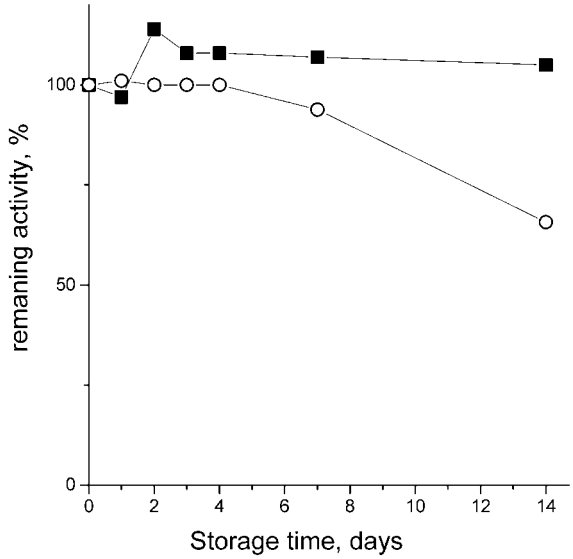

Figure 5. Shelf life of glucose oxidase−Nafion membranes (■) and the casting solution (○) at 4 °C.

ducing principle is the low-potential reduction of hydrogen peroxide with a Prussian Blue-modified electrode.[23,24] Figure 6 presents the calibration for glucose in flow injection mode. It is seen that the biosensor allows the detection of glucose down to the 0.1 μM level. The calibration plot is linear over 4 orders of magnitude of glucose concentration from 0.1 μM to 1 mM (correlation coefficient exceeds 0.9999). An apparent nonlinearity in logarithmic plots (Figure 6) is because at zero glucose concentration the calibration graph does not approach the origin. The FIA response to the solution with zero glucose concentration was not observed. The background current density generated by the glucose biosensor at 0.0 V was constant in the range 10−20 nA cm^{-2}, which was even lower than the FIA response to 10^{-7} glucose. The sensitivity of FIA glucose detection, which has been calculated in the linear range up to 1 mM, is 0.05 A M^{-1}cm^{-2}. That is only 10 times lower than the sensitivity of the transducer to hydrogen peroxide used in this biosensor.

The biosensor showed an appropriate operational stability. The calibration curves taken throughout the day of operation with more than 150 assays overlapped with the only possible variation due to temperature change in the laboratory (data not shown).

The advantages of the proposed immobilization protocol over our previously reported nonaqueous enzymology procedure[13,25] are summarized in Table 1. The stabilization by membrane-forming polyelectrolyte provides at least 5 times improved

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Table 1. Analytical Characteristics of the Metal Hexacyanoferrate-Based Glucose Biosensors with Different Protocols for Immobilization of Glucose Oxidase

immobilization protocol	transducer	mode	biosensor sensitivity, A/(M cm^2)	sensitivity ratio (transducer/ biosensor)	biosensor sensitivity, A/(M mg of E)	lowest level detected[a] or detection limit[b], M	biosensor sensitivity to detection limit ratio, A/(M^{2}cm^2)	ref
In Nafion from water−organic mixtures with stabilization by membrane-forming polyelectrolyte	Prussian Blue	FIA	0.05	10	0.5	1×10^{-7} [a]	5×10^5	
same as previous, but without stabilization	Prussian Blue	FIA	0.01	50	0.03	2×10^{-6} [a]	5×10^3	25
poly(vinyl alcohol) 4-vinylpyridine copolymer	Prussian Blue	steady state	1×10^{-3}	500		0.5×10^{-6} [b]	2×10^3	29
additional metal hexacyanoferrate layer	Cr HCF	steady state	4×10^{-3}	250		3×10^{-6} [b]	1×10^3	30
Nafion membrane	Prussian Blue	FIA	4×10^{-4}	1250		5×10^{-5} [b]	8	31
gelatin gel	Co HCF	steady state	3×10^{-5}	30000				32
entrapment in poly(*o*-phenylenediamine)	Prussian Blue	steady state	7×10^{-4}	700				33

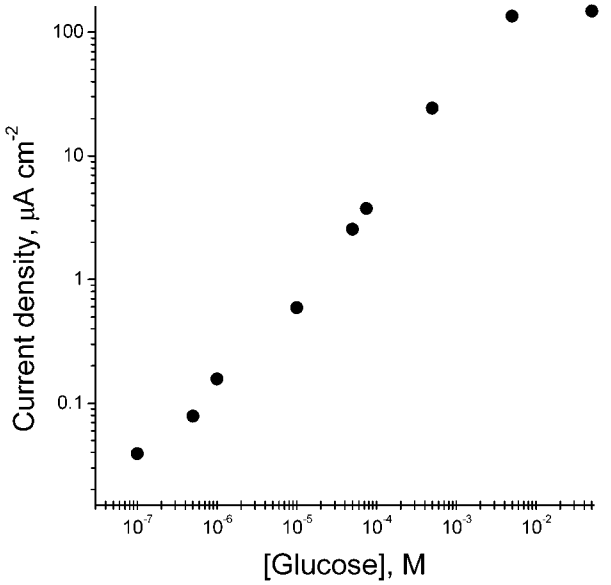

Figure 6. Calibration for glucose-sensitive biosensor: 1.5 mg mL^{-1} glucose oxidase, 0.3% Nafion and 7.4% H_2O in casting solution; flow rate 0.5 mL min^{-1}; $E = 0.0$ V (Ag|AgCl|1 M KCl).

sensitivity and 1 order of magnitude lower detection limit of the resulting biosensors. Taking into account the amount of the enzyme deposited on the electrode surface, one can introduce the sensitivity units per milligram of the protein [A M^{-1}(mg of E)$^{-1}$]. In such units, the sensitivity of the biosensor made according to the proposed approach is ∼20 times higher compared to that of the enzyme electrode elaborated without stabilization (Table 1).

Obviously, sensitivity of an amperometric electrode can be improved by an increase of both roughness of the electrode surface and amount of immobilized enzyme. This, however, increases the lower limit of analyte concentration that can be detected. Thus, another important parameter, which can characterize the amperometric sensor, is the ratio of its sensitivity to the lowest concentration of analyte detected. We would like to discriminate the "lowest level of analyte detected" (LLD) from the "detection limit", because the latter is commonly calculated from calibration curves[34] and, in some cases, extrapolated over several orders of magnitude of analyte concentration. In our opinion, LLD can be determined similarly to some definitions of the detection limit as the reproducible response of the system to the lowest concentration of analyte, which exceeds the response to zero analyte concentration by at least 3 times. We believe it is important that higher LLD, the response has to be significantly dependent on the analyte concentration.

The ratio of sensitivity to LLD of the glucose biosensor made according to the proposed approach is 100 times higher compared to the biosensor elaborated without stabilization (Table 1).

Table 1 also summarizes the analytical characteristics of the five recently reported first-generation glucose biosensors based on transition metal hexacyanoferrates (HCFs) being the most advantageous hydrogen peroxide transducers. Except for Prussian Blue, the high sensitivities to H_2O_2 were reported for cobalt and chromium hexacyanoferrates,[26,27] raising, however, some doubt that these particular hexacyanoferrates are responsible for high electrocatalytic activity of the corresponding modified electrodes.[28] The different immobilization protocols are compared: in poly(vinyl alcohol) grafting 4-vinylpyridine,[29] in an additional metal hexacyanoferrate layer during electrodeposition,[30] covering by Nafion membrane,[31] in gelatin gel,[32] and in poly(*o*-phenylenediamine) during electropolymerization.[33] Table 1 illustrates that that the proposed approach displays a dramatic (100-fold) improvement in sensitivity of the resulting biosensor.

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The sensitivities of the most recently reported first-generation glucose biosensors based on smooth platinum,[35] screen-printed electrodes with rhodium,[36] or metallized carbon pastes[37] are less than 1 mA M^{-1} cm^{-2}, which makes them not competitive with the reported biosensor.

High sensitivities to glucose could be estimated from the calibration plots reported for the biosensors based on platinized glassy carbon,[38,39] referred to as a "porous platinum particle matrix".[38] However, the sensitivities are lower compared to our enzyme electrode, if the relationship between steady state and flow injection assays is taken into account and especially if recalculated relative to the enzyme concentration. Moreover, the rough transducer surface of the experiments of refs 38 and 39 does not allow glucose sensing in the submicromolar range even in batch mode. Even taking the calculated detection limits[38,39] rather than the really achieved LLD, we obtain for enzyme electrodes[38,39] a newly introduced parameter in the frame of $(0.5-1) \times 10^5$ A M^{-2} cm^{-2}, which is 1 order of magnitude lower than that for the developed biosensor (Table 1).

Thus, the proposed approach is the most advantageous one among the known immobilization protocols and provides the most efficient use of the enzyme. In combination with the best hydrogen peroxide transducer (Prussian Blue),[28] the proposed immobilization protocol provides the elaboration of the most advantageous first-generation glucose biosensors.

CONCLUSIONS

We conclude that using a nonaqueous immobilization approach the optimal environment for glucose oxidase in a Nafion membrane can be reached. The inflection points in Figure 2 in a similar concentration region of Nafion confirm that the membrane-forming polyelectrolyte is able to stabilize the enzyme upon its exposure to concentrated organic solvent. Observation of the extremes of both the membrane specific activity (Figure 4) and its stability (Figure 3) at similar enzyme concentrations and the enzyme-to-Nafion ratios indicates the existence of the optimal environment for the enzyme in the polyelectrolyte membrane.

We believed by that improving the nonaqueous immobilization protocol, we were able to improve the reproducibility of the enzyme-containing membranes. Indeed, the activity of the GOx−Nafion membranes is almost 100% reproduced with maximum deviation ∼2%, what is already a precision limit for kinetic investigations.

Considering the advantages of the glucose-sensitive electrode over known biosensors, the most efficient use of the immobilized enzyme was noted. The sensitivity recalculated per milligram of the enzyme was of 0.5 A M^{-1} (mg of E)$^{-1}$. In combination with the stability of the casting solution of about one week, this is highly important in a search for an optimal use of the expensive oxidases in bioanalytical applications.

Combined with the attractive performance of a Prussian-Blue-based hydrogen peroxide transducer, the proposed immobilization protocol provides the elaboration of the most advantageous first-generation glucose biosensors concerning their sensitivity and detection limits. An introduced parameter, the ratio of sensitivity of an amperometric biosensor to the lowest concentration of analyte detected, was 5×10^5 A M^{-2} cm^{-2} for this biosensor.

Since a number of other enzymes were successfully entrapped in Nafion membranes using a previously reported nonaqueous immobilization approach,[13,25] we believe that the proposed protocol is universal for immobilization of enzymes from different classes. This investigation demonstrates how to find an optimal environment for the enzyme in polymer membrane, which provides its maximal efficiency.

ACKNOWLEDGMENT

The financial support through Award from President of Russian Federation, INCO Project IC15CT980906, NATO Linkage Grant HTECH.LG 972789, and INTAS 00-273 is greatly acknowledged. We thank Dr. C. Kelly (New Mexico State University) for helpful comments.

Received for review June 21, 2001. Accepted January 25, 2002.

AC0155409

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