analytical chemistry

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Reagentless Biosensor Based on Glucose Oxidase Wired by the Mediator Freely Diffusing in Enzyme Containing Membrane

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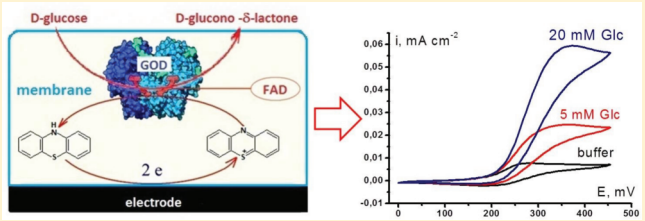
ABSTRACT: Wiring glucose oxidase in the membrane with an immobilized mediator is possible due to the diffusion ability of the latter, if the enzyme containing membrane is formed according to the proposed protocol, including exposing proteins to water−organic mixtures with the high content of organic solvent. In the course of the study, the new glucose oxidase mediator, unsubstituted phenothiazine, was discovered. The diffusion coefficient of the mediator in the resulting membrane is independent of the presence of enzyme. The cyclic voltammograms of the enzyme electrode after appearance of the only glucose in solution obtain a well-defined catalytic shape, which is normally observed for both the enzyme and the mediator in solution. Analytical performances of the resulting biosensor are comparable to the advanced second generation ones, which, however, require covalent linking of the mediator either to the membrane forming polymer or to the enzyme. Even without such covalent linking, the reported biosensor is characterized by an appropriate long-term operational stability allowing reagentless sensing.



The so-called "second generation" biosensors are based on redox mediators aimed to deliver electrons between the enzyme active site and the electrode. The discovery of this type of enzyme electrodes was due to elaboration of novel methods for evaluation of enzyme activity.[1] However, almost at the same time the importance of analytical applications raised an interest to couple the glucose oxidase and the electrode reactions for successive detection of glucose[2] replacing O_2-dependent first generation biosensors.[3,4]

Historically, the first potential mediator for glucose oxidase was Methylene Blue used in a spectrophotometric assay for evaluation of enzyme activity.[5] Despite several attempts to use quinones, azines, metal complexes,[2,6] and even organic metals[7] as mediators, wide practical applications of the second generation electrochemical glucose biosensors started from the discovery of ferrocenium (oxidized ferrocene) as a valuable competitor relative to oxygen, the natural glucose oxidase substrate.[8]

Among the advantages of the second generation oxidase-based biosensors are (i) operation in oxygen free media and (ii) a possibility for coulometric detection. The 10-fold excess of glucose over oxygen in blood makes the latter mode unsuitable for first generation biosensors.

The main disadvantage of the common second generation biosensors is the poisoning of an analyzing object with the mediator, which, for instance, makes impossible biosensor implantation. Accordingly, special efforts were put to immobilize the mediators. Reagentless second generation biosensors were made by immobilization of glucose oxidase in ferrocene-modified polypyrrole[9] as well as other polymers.[10,11] Covalent linking of the ferrocene derivative to the enzyme molecule[12,13] seems to be even more complicated and less successful. Phenothiazine linking to glucose oxidase is also reported displaying catalytic cyclic voltammograms of the enzyme electrode.[14] Enzyme immobilization in hydrogels containing Os complexes with one of the ligands covalently linked to the polymer chain[15−18] deserves the special attention particularly due to both its wide use in fundamental science and important practical applications.

Simplification of the biosensors, required for their mass production, dictates a search for mediator immobilization without covalent linking either to membrane forming polymer or to the enzyme. On the basis of the knowledge that polyelectrolyte membranes are able to retain charged redox active compounds,[19] the ferrocene containing Nafion membrane was coupled with glucose oxidase film to form a reagentless biosensor.[20] An attempt to immobilize both the enzyme and the mediator in the same membrane[21] caused the decrease of sensitivity obviously due to enzyme immobilization.

As we reported, it is possible to retain properties of the polymer membranes even after enzyme immobilization carrying out the latter from water−organic media with the high content of organic solvent.[22−24] Indeed, in such a case, membrane formation occurs from the favorable medium without excessive dilution with water.

Here we report on the successful immobilization of both the enzyme and the mediator avoiding covalent linking of the latter. The membrane containing both the enzyme and the mediator in the presence of only glucose in solution displays the

Received: November 17, 2011
Accepted: December 28, 2011
Published: December 28, 2011

dx.doi.org/10.1021/ac203056m | *Anal. Chem.* 2012, 84, 1220−1223

catalytic-type cyclic voltammograms similar to those observed previously for both glucose oxidase and the advanced mediator in solution. The second generation biosensor with enzyme and mediator both immobilized is stable enough for multiple measurements and even continuous monitoring.

EXPERIMENTAL SECTION

Materials. Experiments were carried out with Milli-Q water from a Millipore Milli-Qsystem. All inorganic salts, siloxans, and organic solvents were obtained at the highest purity from Reachim (Moscow, Russia) and used as received. D-Glucose was purchased from ICN Biomedicals. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger* (lyophilized powder, activity 269 U mg^{-1}) was purchased from Sigma, Germany.

Planar electrodes were made by screen printing (Screen Printer SCF-550, Technical Industrial Co. Ltd., Hong Kong) on polyester films. For both the working and auxiliary electrodes, the carbon inks C10903P14 and C2030519D4 (Gwent, U.K.) providing reversible voltammograms of hexacyanoferrate in neutral media were chosen. The working electrode diameter was 1.9 mm.

Instrumentation. For electrochemical measurements, a μAutolab type III bipotentiostat system (Autolab, EcoChemie, Netherlands) was employed. Electrochemical experiments were made in a three-compartment electrochemical cell containing a platinum net auxiliary electrode and a Ag|AgCl reference electrode in 1 M KCl. Cell construction allowed deaeration of the working electrode space.

The flow injection system consisted of a Cole Parmer (Vernon Hills, IL) peristaltic pump (7519-10), homemade flow-through wall-jet cell with a 0.5 mm nozzle positioned at 1−2 mm distance from the surface of the disk electrode, homemade injector, and a PalmSens electrochemical interface interfaced to an IBM PC. Flow rates used were 0.7 mL min^{-1}. In flow injection analysis (FIA) experiments, the peak current density values were taken for data treatment, the sample volume was 50 μL, and the working electrode potential was 300 mV (vs Ag|AgCl|0.1 M KCl reference). The carrier solution in the FIA experiments was 0.05 M phosphate buffer pH 7.0 containing 0.15 M NaCl as the supporting electrolyte. The film thickness was measured with a Talystep step profilometer (Taylor Hobson) on highly oriented pyrolytic graphite (HOPG) (GRBS/1.2).

Methods. Glucose biosensors were made as follows. Glucose oxidase aqueous solution (10 mg mL^{-1}) was suspended in an isopropanol solution of triethoxysiloxane (γ-aminopropyl-, vinyl-, or phenyl-triethoxysiloxane) (0.1−1.5%) containing azines in the range from 1 to 50 mM. The resulting mixture (2−3 μL) was applied with a syringe onto the working electrode covering its entire surface and dried in a refrigerator (4 °C).

RESULTS AND DISCUSSION

The concept of biomolecule immobilization from water−organic mixtures with the high content of organic solvent is based on the knowledge that enzymes retain their activity after being exposed to such media.[22,23] On the other hand, concentrated organic solutions are favorable for formation of uniform membranes due to (i) use of real polymer solutions[22,23] or (ii) carrying out condensation of siloxanes[24] avoiding in both cases excessive dilution of the reaction medium with water.

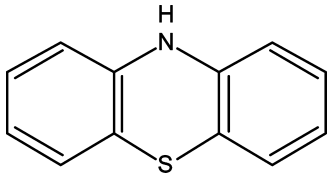
Isopropanol was chosen as the optimal organic solvent for enzyme immobilization, as for formation of polyelectrolyte or siloxane gel membranes. Accordingly, the remaining activity of glucose oxidase after exposure to water−isopropanol solutions was investigated (data not shown). As found, the maximum of the remaining activity (at the level of 85−90%) occurs at 15% water content, which coincides with the water−isopropanol azeotrope. All further experiments were carried out using this particular mixture.

We investigated both the activity and stability of the mediators in enzyme containing membranes. The Nafion analogue (perfluorosulfonated ionomer), due to its cation exchange nature to retain positively charged mediators,[19,25,26] was investigated with immobilized thionine (as a mediator) and glucose oxidase. Even in the presence of the immobilized enzyme, the electroactivity of the azine obeys the diffusion limiting electrochemistry (peak current proportional to the square root of the sweep rate and the peak potential independent of it) (data not shown). Hence, even after enzyme immobilization, the Nafion-type membrane is still able to retain freely diffusing mediator.

For charged analytes, it is important to immobilize enzyme into neutral membranes because the charged one obviously affect analytical performances of the resulting biosensor. Accordingly, we investigated glucose oxidase immobilization in a gel of siloxanes using novel protocol based on water−organic mixtures with the high content of organic solvent reported for lactate oxidase.[24] Obviously, to provide the mediator immobilization in such a membrane, the water insoluble azines were chosen.

We tested unsubstituted phenothiazine, phenoxazine, and their oligomers. Among them the only phenothiazine (Scheme 1) displayed electroactivity when cycled in neutral solutions. Figure 1 displays cyclic voltammograms of the electrode modified with phenyl-siloxane gel containing both the enzyme and the mediator (casting solution contained 1.0% siloxane, 10 mM phenothiazine, 1.5 mg mL^{-1} GOD). As seen, similarly to the Nafion-type membranes (above), at moderate sweep rates the cyclic voltammograms obey the behavior of the reversible electrochemical reaction, i.e., limiting by diffusion of the depolarizer. Indeed, the peak current is proportional to the square root from the sweep rate (Figure 1, inset), and the peak potential is independent of it.

Scheme 1. Chemical Formula of Phenothiazine



The redox mechanism of phenothiazine is a two electron process. In cyclic voltammograms of the mediator in a γ-aminopropyl-triethoxysilane membrane, the two sets of peaks are clearly observed. Dependence of the common peak potentials on solution pH points to the two electron−two proton reaction. Despite the oxidized form of phenothiazine is charged and likely water-soluble, the cyclic voltammograms of the mediator in siloxane gels are rather stable, displaying more than 90% of the initial peak current value even after 100 cycles (data not shown).

dx.doi.org/10.1021/ac203056m | *Anal. Chem.* 2012, 84, 1220−1223

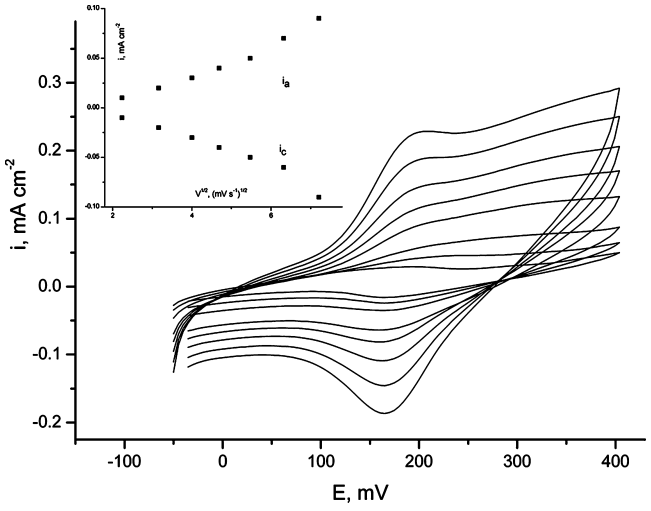


Figure 1. Cyclic voltammograms of phenyl-triethoxysilane gel membrane containing both glucose oxidase and phenothiazine; 0.05 M phosphate buffer (pH 7.0) with 0.15 M NaCl. Inset: peak current dependence on the square root of the sweep rate.

Discussing diffusion peculiarities of the mediator, we made an attempt to evaluate the diffusion coefficient of phenothiazine in siloxane gel. The mediator concentration in the membrane was calculated considering the final volume of the gel. The membrane thickness (measured using profilometry) was at the level of 1 μm. The diffusion coefficient was calculated from the slope of the peak current vs the sweep rate dependence using formulas for reversible electrochemistry.[27]

For correct evaluation of the diffusion coefficient in a membrane, it is necessary to carry out experiments at low mediator concentrations. With an increase of the latter, the diffusion coefficient cannot be measured correctly and its apparent value starts to be dependent on mediator content. Indeed, the substantial decrease of the apparent diffusion coefficient with an increase of the azine concentration in the membrane was recorded.[25] Unfortunately, we were unable to decrease the phenothiazine concentration in the casting solution below 1 mM because high capacitive currents masked mediator electroactivity. At this concentration level, a 2.5 times increase of the phenothiazine concentration caused almost a 5 times decrease of the apparent diffusion coefficient. Hence, the evaluated diffusion coefficient value with a 10^{-14} cm^2 s^{-1} order of magnitude is unrealistic.

However, it was possible to investigate whether the presence of the enzyme in the membrane affects the diffusion peculiarities of the mediator. As was found, the phenothiazine diffusion coefficient in the enzyme free γ-aminopropyl-triethoxysilane gel is not higher compared to the enzyme containing membrane. Hence, the enzyme immobilized according to the proposed protocol does not affect the diffusion properties of a mediator in the resulting membrane.

Obviously, for aqueous electrochemistry, the water-insoluble redox compound can be immobilized onto the electrode surface by a simple dip-coating from its organic solution. However, in this case, the modified electrode behaves as containing an adsorbed redox couple (the peak current is proportional to the sweep rate). The above observations point to the diffusion ability of phenothiazine in siloxane gel even containing immobilized enzyme.

Siloxane gels with immobilized glucose oxidase and the mediator were optimized considering concentrations of all components to provide both the highest stability and the highest response. The optimal membranes were investigated both in a flow-injection system with amperometric detection and by cycling voltammetry. As shown in Figure 2, after

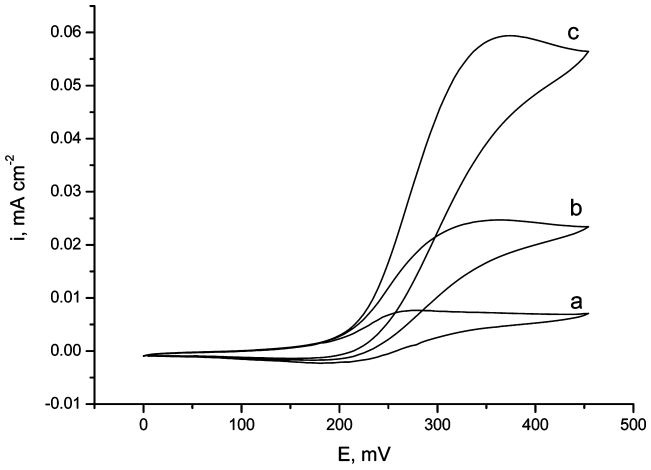


Figure 2. Cyclic voltammograms of a vinyl-triethoxysilane−GOD−phenothiazine gel membrane (casted from 0.3% siloxane, 5 mM phenothiazine, 1.5 mg mL^{-1} GOD) in the absence (a) and in the presence of 5 mM (b) and 20 mM (c) glucose in solution; 0.05 M phosphate buffer (pH 7.0) with 0.15 M NaCl; sweep rate 2 mV s^{-1}.

appearance of the only glucose in solution due to its penetration in membrane, the cyclic voltammograms obtain a catalytic shape. Such a well-defined shape normally observed for both the enzyme and the mediator in solution[8] also indicates the diffusion ability of phenothiazine in a membrane.

We note that being water-insoluble, phenothiazine was unknown as the mediator for glucose oxidase. Indeed, when immobilized by dip-coating from its organic solution, phenothiazine does not show mediation ability even in the presence of both glucose and its oxidase in solution (data not shown).

Analytical performances of the elaborated second generation biosensor based on glucose oxidase and phenothiazine immobilized into siloxane gel were investigated in the flow-injection system. Sensitivity of the biosensor in FIA mode is at a level of 2 mA M^{-1} cm^{-2}, which taking into account experimental conditions, and particularly dispersion coefficient, is at a level of the most advanced system based on Os containing hydrogel with one of the ligands covalently bound to the polymer matrix.[28] The lower detection limit for glucose (0.015 mM) is among the lowest ones for second generation biosensors.

The crucial point for reagentless second generation biosensors is their operational stability. Accordingly, the latter for the elaborated biosensor was investigated. As seen in Figure 3, the wall-jet detector equipped with the biosensor remains at its initial response (precisely, 98 ± 1%) after 50 injections. The inset of Figure 3 also shows the fast response of the biosensor. Hence, the elaborated second generation biosensor with immobilization for both the enzyme and the mediator is stable enough for multiple analyses and even continuous monitoring.

dx.doi.org/10.1021/ac203056m | *Anal. Chem.* 2012, 84, 1220−1223

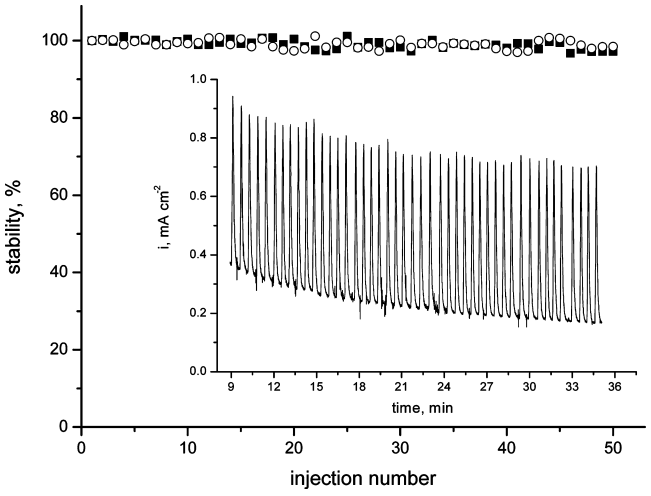


Figure 3. Operational stability of the γ-aminopropyl-triethoxysilane based biosensor (casting solution 1.0% γ-NH_2PrSil, 50 mM phenothiazine, 1.5 mg mL^{-1} GOD) in FIA mode. Inset: response of the biosensor integrated in the wall-jet cell to continuous injections of 5 mM glucose; 0.05 M phosphate buffer, pH 7.0, with 0.15 M NaCl, flow rate 0.8 mL min^{-1}, potential 0.300 V (vs Ag/AgCl‖0.1 M KCl).

CONCLUSIONS

We conclude that the application of the improved immobilization protocol including exposure of the enzymes to water−organic mixtures with a high content of organic solvent for second generation biosensors allows elaboration of cheap reagentless biosensors avoiding covalent linking of the mediator either to the membrane forming polymer or to the enzyme. The biosensors are characterized by operational stability suitable for multiple detections.

The novel immobilization protocol allows wiring of glucose oxidase with the water insoluble mediator able to diffuse in the enzyme containing membrane. In the course of the study, the new glucose oxidase mediator, unsubstituted phenothiazine, was discovered.

Both electrochemical and analytical properties of the reported enzyme electrode are at the level of the advanced second generation biosensors. We strongly believe that the simplicity of manufacturing, on one hand, and the advanced resulting properties, on the other, provide wide application of the reported approach for both fundamental research and practical applications.

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