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Self-encapsulation of oxidases as a basic approach to tune the upper detection limit of amperometric biosensors

Arunas Ramanavicius,*[a] Asta Kausaite[a,b] and Almira Ramanaviciene[a]

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This study describes a new, basic procedure for the tuning of some analytical parameters of enzymatic biosensors that are based on hydrogen peroxide-producing oxido-reductases. An amperometric biosensor based on glucose oxidase (GOx) (EC 1.1.3.4) from *Penicillum vitale*, immobilized on a carbon rod electrode by cross-linking with glutaraldehyde, was exploited as a model system for demonstration of the approach described here. Such an important analytical parameter as the upper detection limit was dramatically changed by the formation of a polypyrrole conducting polymer layer by the GOx-induced polymerization of polypyrrole (Ppy). An increase in the upper detection limits for differently modified electrodes was estimated by calculation of the apparent Michaelis–Menten constant [$K_{M(app)}$]. A significant increase in the long-term stability of the GOx-based electrode modified by Ppy (GOx/Ppy) was detected compared with that of an unmodified one. Further application of this approach, based on the self-encapsulation of glucose oxidase and other oxidases, is predicted for such biosensors where extension of the detection rate as well as $K_{M(app)}$ are required.

Introduction

Amperometric biosensors based on enzymes such as oxido-reductases belong to one of the most widely used classes of biosensors. Some oxido-reductases (*e.g.* glucose oxidase,[1–3] alcohol dehydrogenase,[4] *etc.*) are frequently applied in the design of biofuel cells powered by alcohol[4] and/or glucose.[1–3] Enzyme immobilization is a required procedure in the design of the above-mentioned bioelectronic devices because it allows application of immobilized enzymes for continuous operations.[5] Furthermore, enzyme immobilization provides many advantages such as enhanced stability, easy separation from the reaction mixture, possible modulation of the catalytic properties, and easier prevention of microbial growth. To some extent, enzyme immobilization allows the variation and adaptation of experimental parameters, which is one of the major topics for construction of amperometric biosensors.[6–8] For some amperometric biosensors (*e.g.* assigned for the detection of glucose), extension of the linear range is sought that can be easily reached by an increase of the apparent Michaelis–Menten constant, $K_{M(app)}$. After immobilization of an enzyme, the $K_{M(app)}$ of the biosensor might be too low; additional procedures to increase $K_{M(app)}$ could then be investigated. Various strategies are applied to increase the $K_{M(app)}$ of such biosensors, but the most popular of them are based on the application of membranes and/or polymeric layers with advanced diffusion properties. However, the introduction of optional membranes and/or polymeric layers usually requires additional procedures that are mainly manual and time consuming. Moreover, such manipulations are sometimes not possible, especially if the biosensors are miniaturized and/or integrated into complicated flow-through designs (*e.g.* lab-on-a-chip devices). On the other hand, in practical terms, application of the usual methods for introduction of an additional diffusion layer[9–11] does not allow precise adjustment of $K_{M(app)}$ for each biosensor, because fine adaptation of the optimal polymeric layer for each individual biosensor is reqired.

Conducting polymers are often applied for the immobilization of various enzymes.[12] After enzyme immobilization within conducting polymers, parameters such as $K_{M(app)}$ are changed significantly, thus causing an increase in the upper detection limit of the biosensors created. Among the conducting polymers used, polypyrrole (Ppy) has found most applications, especially as an immobilization matrix in the design of various catalytic biosensors based on the catalytic action of enzymes;[13,14] also, it is often used in the design of immunosensors[15–17] and DNA sensors.[18,19] It was also shown that conducting polymers such as polypyrrole and substituted polythiophene[20] are able to transduce directly the analytical signals generated by some redox enzymes, *e.g.* quinohemoprotein alcohol dehydrogenase[21] and even glucose oxidase.[20] To enhance its functions, polypyrrole can be easily modified by metal nanoparticles (including gold[22] and silver[23] nanoparticles), carbon nanotubes,[24] covalently attached red-ox groups,[25] proteins,[19] or molecularly imprinted by low-[26] and high-molecular weight[27] analytes or even spores of bacteria.[28] On the other hand, Ppy protects electrochemical biosensors from anionic electrochemically-interfering materials.[17] Moreover, polypyrrole is a promising material for the design of implantable biosensors because of its relative stability[29] and biocompatibility.[30–32]

[a]*Center of Nanotechnology and Material Science, Faculty of Chemistry, Vilnius University, Naugarduko 24, 03225 Vilnius, Lithuania*
[b]*Laboratory of Immunoanalysis and Nanotechnology, Institute of Immunology of Vilnius University, Moletu pl. 29, 08409 Vilnius, Lithuania*

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The application of conducting polymers in the design of catalytic biosensors was started by the entrapment of glucose oxidase within polypyrrole.[33,34] The incorporation of enzymes into Ppy matrices provides a very convenient electrochemical procedure. The embedding of enzymes within a Ppy film prevents the enzyme from being leached out, while at the same time maintains the accessibility of the catalytic sites due to the permeability of the film to analytes.[35] Pulse techniques for the electrochemical deposition of polymer films on electrode surfaces enabled the concentration of entrapped enzyme to be increased within thin layers of Ppy.[36] Furthermore, the enzyme activity is usually detected by characterization of final reaction products or red-ox mediators using amperometric or potentiometric methods. Electrochemical polymerization is currently the most widely used method for applications of Ppy in biosensorics because it offers many advantages: it can produce an easily prepared, high-conductivity Ppy, and can enable *in situ* coating of Ppy on rather complex geometries. However, methods of electrochemical Ppy deposition have some limitations too: (i) the polymerization reaction should be conducted in oxygen-free solution, and (ii) more-or-less stable analytical parameters can be observed only after a Ppy swelling period, which can usually be up to several days.[21] Such limitations significantly reduce the applicability of Ppy-based systems in the design of amperometric biosensors. Chemical synthesis of Ppy under mild polymerization conditions[37] can be foreseen as a method that allows lessening of the previously mentioned drawbacks. The chemical method of polypyrrole synthesis is based on the initiation of polymerization by oxidative compounds.[38] In this way, Angeli firstly synthesized polypyrrole in 1916.[39] Polypyrrole synthesized by conventional chemical methods is insoluble in usual solvents because of strong inter-chain interactions.

Our previous studies demonstrated that GOx dissolved in the polymerization bulk might be encapsulated within Ppy by the formation of nanoparticles[40] that exhibit significantly different catalysed reaction kinetics, compared with the native enzyme, and therefore can be applied in the design of amperometric biosensors that allow the detection of glucose over a wider concentration interval compared with biosensors based on native GOx immobilized in a similar way.[41] These studies encouraged us to apply this polymerization/self-encapsulation method for increasing the analyte detection intervals of a very convenient glucose (Glc) biosensor. The aim of this study was to modify a cross-linked, GOx-based electrode by self-encapsulation of GOx within Ppy in order to show a new possibility of changing the catalytic parameters [$K_{M(app)}$ and V_{max}] of an amperometric biosensor based on this electrode and to increase its upper detection limit.

Experimental

Chemicals

Analytical grade chemicals and triply distilled water were used to prepare the solutions. Glucose oxidase from *Penicillum vitale* was purchased from BIOTUL (Kiev, Ukraine), and pyrrole and phenazine methosulfate (PMS) were purchased from SIGMA (Berlin, Germany).

Electrode pre-treatment and modification by GOx and Ppy

Carbon rod electrodes 'Ultra F purity' 3 mm in diameter were obtained from Ultra Carbon Division of Carbon USA, RAVEN-M. Carbon rod electrodes were sealed into epoxy to prevent contact of the electrode side surface with the solution. The working surface area of the graphite electrodes was 7 mm^2. Graphite electrodes modified with GOx (GOx-electrodes) were prepared as follows: first, rods of spectroscopic graphite were cut and polished on fine emery paper and then polished by Al_2O_3 slurry (grain size 0.1 μm), followed by rinsing the electrode surface with ethanol and distilled water. Electrodes were dried at room temperature before coating with the enzyme.

Electrode modification by GOx

During preparation of the GOx-coated electrodes, 3 μl of 40 mg ml^{-1} enzyme solution were deposited on the electrode and later water was evaporated at room temperature by intensive ventilation. Then electrodes were stored for 24 h over a 5% solution of glutaraldehyde at 4 °C in a closed vessel as described previously.[42] Prior to electrochemical measurements, the electrodes where thoroughly washed with distilled water to remove non-cross-linked enzyme.

Modification of GOx-coated electrode with a Ppy layer

For preparation of electrodes possessing new analytical properties, electrodes modified with GOx were immersed into three different 0.05 M sodium acetate buffer solutions, pH 6.0. The first of them contained 50 mM of glucose and 300 mM of pyrrole (polymerization solution), the second contained 50 mM of glucose (control solution 1), and the third contained 300 mM of pyrrole (control solution 2). Prior to electrochemical measurements the electrodes were thoroughly washed with distilled water. To allow equilibration of mutarotation, the solutions of glucose were prepared at least 24 h before use.

Preparation of control electrodes: modification of GOx-coated electrode in control solutions without some components required for pyrrole polymerization

Two types of solutions, which do not contain one of the chemicals crucial for polymerization (glucose and pyrrole), were used to investigate the influence of chemicals that were used for polymerization on the kinetic parameters [$K_{M(app)}$ and V_{max}] of the GOx-coated electrode. The first control solution does not contain glucose; the second was free of pyrrole.

Electrochemical detection of analytical signal

All electrochemical measurements were performed by potentiostat-galvanostat PGSTAT 30 with GPES3 v3.2 software ECO-Chemie/Autolab (Utrecht, Netherlands). A three-electrode circuit was applied for registration of the amperometric signal. The GOx- or GOx/Ppy-modified carbon electrode was used as the working one; Ag/AgCl as the reference and a 2 cm^2 Pt electrode was used as the auxiliary one. Electrochemical signals were detected in sodium acetate buffer with 0.1 M KCl,

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pH 6.0. The solution in the electrochemical cell was mixed at 120 rpm.

Special conditions used for the electrochemical detection of hydrogen peroxide

Reaction was performed at +800 mV *vs.* Ag/AgCl in the presence of different glucose concentrations.

Special conditions for the electrochemical detection of PMS

The reaction was performed at +300 mV *vs.* Ag/AgCl in the presence of 10 mM of PMS and different glucose concentrations.

Special conditions used for the determination of pH dependence

GOx- and GOx/Ppy-modified electrodes were tested in different buffers with fixed pH. Electrochemical detection of the analytical signal was performed at room temperature at +300 mV *vs.* Ag/AgCl in the presence of 10 mM of PMS and 30 mM of glucose.

Special conditions in stability test

Between measurements, the GOx- and GOx/Ppy-modified electrodes were stored at 4 °C in a closed vessel hanging over phosphate buffer solution. Electrochemical detection of the analytical signal was performed at room temperature at +300 mV *vs.* Ag/AgCl in the presence of 10 mM of PMS and 30 mM of glucose.

Measurements of Ppy thickness over GOx

An atomic force microscope, Bioscope II Veeco (Santa Barbara, USA), was used to measure the thickness of the formed GOx and GOx/Ppy films.

Results and discussion

The formation of polypyrrole induced by GOx dissolved in the polymerization bulk solution consisting of glucose and pyrrole has been described in detail elsewhere.[37] Formation of the core/shell GOx/Ppy nanoparticles[40] that exhibited GOx enzymatic activity with significantly increased $K_{M(app)}$ and decreased V_{max}, in comparison with the parameters of native GOx, was detected.[41] These findings encouraged us to transfer this basic GOx modification technology towards the technology for tuning of the kinetic parameters of oxidase-based biosensors. The catalytic activity of GOx was exploited in this study for the generation of hydrogen peroxide [reaction (1)], which initiates the polymerization of pyrrole, followed by deposition of a Ppy layer over a cross-linked GOx layer (Fig. 1). Referring to our further investigations, the main precursors for the initiation of the pyrrole polymerization reaction are: (i) hydrogen peroxide (as an initiator of the polymerization reaction itself), and (ii) gluconic acid as a medium that reduces the pH towards the optimal pH for this polymerization reaction. All mentioned precursors were produced during the catalytic action of GOx [reaction (1)] and the following hydrolyzation of the reaction products [reaction (2)].

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconolactone} + H_2O_2 \quad (1)$$

$$\text{Gluconolactone} + H_2O \rightarrow \text{Gluconic acid} \quad (2)$$

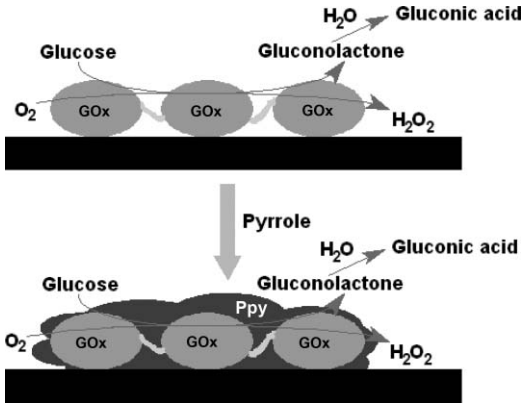

Fig. 1 Principal scheme illustrating modification of the GOx-based electrode by Ppy.

For confirmation of our insight that the catalytic action of an enzyme might be exploited to increase the linear range of the system, a basic biosensor design was selected, which was based on an enzyme cross-linked by glutaraldehyde on the surface of a carbon rod electrode according to our previously elaborated and described methodology.[42] These and very similar structures are common in enzyme-based biosensors[43] and other bioelectronics devices; however, sometimes the dilution of an analyte-containing solution is required since the $K_{M(app)}$ value of such a biosensing system is significantly lower compared with the concentration of analyte in the sample.[44] In such cases, the introduction of additional diffusion limitations will be a possible way to increase the linear range of this biosensing system to result in an increase of the $K_{M(app)}$ value. There are several strategies to achieve this goal: (i) externally-prepared diffusion membranes are mechanically overlaid on top of the enzymatic layer,[45] and (ii) a polymeric layer is chemically formed and/or deposited by solvent casting on the enzymatic layer. However, both strategies have significant drawbacks, for example: (i) in the case of membrane application, externally-prepared diffusion membranes have discrete diffusion values, and therefore their application makes it difficult to achieve the required $K_{M(app)}$ value exactly; and (ii) deposition of the polymeric layer is also usually not well controlled since solvent casting and/or the usual chemical polymerization does not allow exact fixation of required $K_{M(app)}$ value. Besides, a relatively broad distribution in $K_{M(app)}$ is common when a number of electrodes are prepared by this procedure. The data presented in Fig. 2A and Fig. 2B illustrate the new approach proposed in this article upon formation of the polymeric layer by the GOx-induced polymerization of pyrrole, together with its advantages over other previously mentioned methods: it allows simple termination of the polymerization reaction once the required $K_{M(app)}$ value is reached and the reaction can be prolonged if the desired $K_{M(app)}$ has not yet been achieved. It means that deposition of the Ppy layer continues while the GOx electrode is present in the polymerization solution. According to calculations presented in Fig. 2B the $K_{M(app)}$ can be extended from 5.8 up to around 250 mM, allowing $K_{M(app)}$ to be increased by 43 times. For the present study the GOx from *Penicillum vitale*

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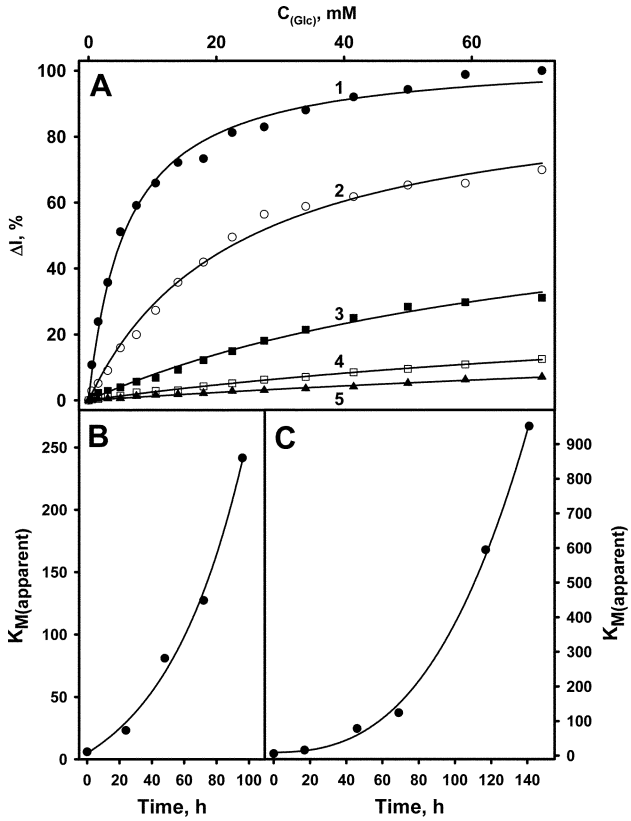

Fig. 2 (**A**) Glucose calibration curves obtained with a GOx-modified carbon electrode, before treatment (curve 1) and after treatment with 300 mM pyrrole and 50 mM glucose solution (curves 2, 24 h; 3, 48 h; 4, 72 h; and 5, 96 h). The *y*-axis corresponds to the variation of the electrode current. (**B**) Calculated $K_{M(app)}$ for glucose *vs.* duration of incubation in pyrrole and glucose solution. Amperometric measurements presented in plots **A** and **B** were performed at +800 mV *vs.* Ag/AgCl in 50 mM sodium acetate buffer with 100 mM KCl, pH 6.0. (**C**) Calculated $K_{M(app)}$ for glucose *vs.* polymerization time. Experiments presented in plot **C** were performed at +300 mV *vs.* Ag/AgCl in 50 mM sodium acetate buffer with 100 mM KCl and 10 mM PMS, pH 6.0.

was selected because this enzyme exhibits the lowest K_M among other available glucose oxidases.[41] Because of its excellent stability, GOx is applied in some commercial systems for the determination of glucose. Usually, a structure based on several diffusion membranes is applied to extend the upper detection limit due to reduced diffusion of the substrate.

The calibration of such a device after deposition of the biosensing part is required because all of the mentioned structures exhibit significantly different $K_{M(app)}$ values. The model system used in this study was based on the same enzyme (GOx from *Penicillum vitale*), and it initially exhibited a relatively low $K_{M(app)}$ value (5.8 mM) calculated for glucose (Fig. 2A, curve 1; Fig. 3A, curve 1; Fig. 4A, curve 1). For this reason, application of the GOx enzyme was optimal for this study in order to register a significant increase in $K_{M(app)}$ of up to 250 mM (Fig. 2A, curve 5). Such a significant extension of the analyte detection interval is especially useful for the detection of glucose concentrations in food and beverage samples. After 96 h of polymerization, formation of a 100–200 nm Ppy layer over the deposited GOx was verified by mean values obtained by atomic force microscopy.

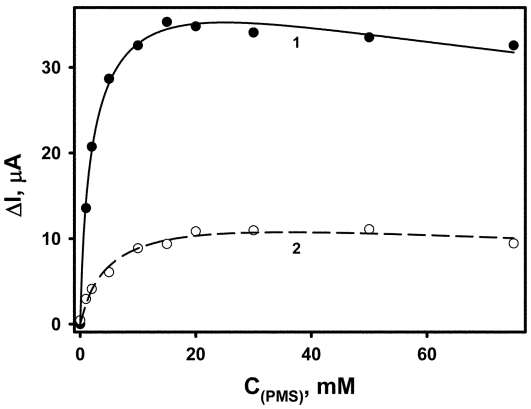

Fig. 3 PMS calibration curves obtained with a GOx-modified carbon electrode (curve 1), and for a similar electrode treated for 24 h with 300 mM pyrrole and 50 mM glucose solution (curve 2). Amperometric detection was performed at +300 mV *vs.* Ag/AgCl in 50 mM sodium acetate buffer with 100 mM KCl and 30 mM of glucose, pH 6.0.

The detection of hydrogen peroxide formed during the catalytic action of GOx on the carbon electrode gives relatively low currents even at very high potentials (+800 mV *vs.* Ag/AgCl). To reduce the detection potential and increase the amperometrically registered current, red-ox mediators can be applied. For this, we tested the performance of these GOx- and GOx/Ppy-based systems with the soluble red-ox mediator phenazine methosulfate (PMS). In the presence of PMS, an amperometric signal at +300 mV was higher by two orders of magnitude compared with that in the absence of PMS at +800 mV, because the oxidation of H_2O_2 at carbon occurs at a higher voltage and is significantly slower compared with PMS. When the additional red-ox mediator (PMS) was applied, a 10–15% increase in the $K_{M(app)}$ values obtained was registered for glucose (Fig. 2C) compared with similar measurements without PMS (Fig. 2B). Moreover, the application of PMS significantly increased amperometric signal and increased the sensitivity of the bioanalytical system. This allowed the precise measurement of markedly reduced GOx-catalysed reaction rates, which were detected after long-term (*e.g.* 140 h) polymerization periods. In the presence of PMS it was detected that $K_{M(app)}$ for the GOx/Ppy-modified electrode increased up to almost 1000 mM (Fig. 2C). This means that GOx has catalysed self-encapsulation, resulting in a 172-fold increase of $K_{M(app)}$ when compared with $K_{M(app)}$ of the GOx-modified electrode.

This difference might be related to the different diffusion properties of PMS compared with those of the natural electron acceptor oxygen: PMS is a bigger molecule compared with oxygen and it is also positively charged. Therefore the diffusion of PMS within the Ppy layer,[40] which is polyionite, is more complicated. However, such insignificant differences (only 10–15%) show that diffusion hindrances for glucose are playing the most important role in the increase of $K_{M(app)}$. To estimate the influence of the Ppy layer on the permeability of PMS, the $K_{M(app)}$ value for PMS was calculated for two electrodes: one modified with GOx, and the second modified with GOx/Ppy (Fig. 3). For PMS it was found that the $K_{M(app)}$ with the GOx/Ppy electrode is three times higher than that of the GOx-modified electrode. Such a difference is comparable to the differences registered for the $K_{M(app)}$ of glucose (Fig. 2). Similar changes in the $K_{M(app)}$ values

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for PMS and for glucose illustrate that the diffusion limitations of Ppy detected for PMS are very similar to those detected for glucose.

The data presented in Fig. 2A and 2B show that the polymerization reaction takes place over a long period of time. In this reaction, the glucose and pyrrole used, as well as the hydrogen peroxide and gluconic acid formed, could theoretically have a significant influence on the kinetic parameters of the GOx-coated electrode for several reasons: (i) the possible reaction of the initial materials (glucose and pyrrole) as well the reaction of the products formed (hydrogen peroxide and gluconic acid) with the protein shell and active centre of the enzyme; (ii) the fouling and electrochemical inactivation of the electrode surface; and (iii) the detachment of the immobilized enzyme from the surface of the electrode. To study the influence of such effects upon kinetic parameters, two theoretically possible types of control solutions were tested: a first solution, without pyrrole, which is essential for the polymerization reaction (Fig. 4); and a second solution, without glucose, which is crucial for the formation of hydrogen peroxide – the initiator of the pyrrole polymerization reaction (Fig. 5).

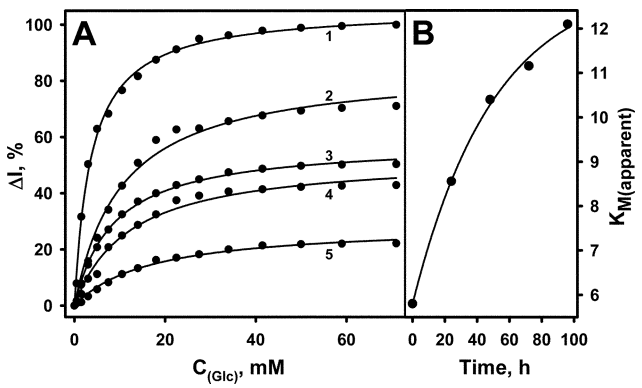

Fig. 4 (**A**) Glucose calibration curves obtained with a GOx-modified carbon electrode after different treatment durations with control solution 1 containing 50 mM of glucose but not containing any pyrrole (curves 1, 0; 2, 24 h; 3, 48 h; 4, 72 h; and 5, 96 h). (**B**) Calculated $K_{M(app)}$ *vs.* duration of incubation in 50 mM glucose solution. Amperometric detection was performed at +800 mV *vs.* Ag/AgCl in 50 mM sodium acetate buffer with 100 mM KCl, pH 6.0.

The absence of pyrrole resulted in a smaller increase of $K_{M(app)}$, by over 20 times compared with that obtained using 50 mM of glucose and 300 mM of pyrrole (Fig. 2B). On the other hand, in the presence of all components (glucose and pyrrole) an exponential increase in $K_{M(app)}$ was found (Fig. 2B), whereas in the absence of pyrrole a hyperbolic increase of $K_{M(app)}$ was found (Fig. 4B). In the presence of all components (glucose and pyrrole) in the polymerization bulk solution, the hydrogen peroxide formed initiated the polymerization of pyrrole, and gluconic acid reduced the pH. Both factors facilitated an exponential deposition of Ppy on the interface between the GOx layer and the solution with a resulting increase in $K_{M(app)}$ (Fig. 2B).

If glucose was absent from the polymerization bulk solution, in contrast to the previously discussed cases (Fig. 4B), $K_{M(app)}$ decreased twice (Fig. 5B) due to slow degradation of the GOx layer, which became thinner and diffusion through it became

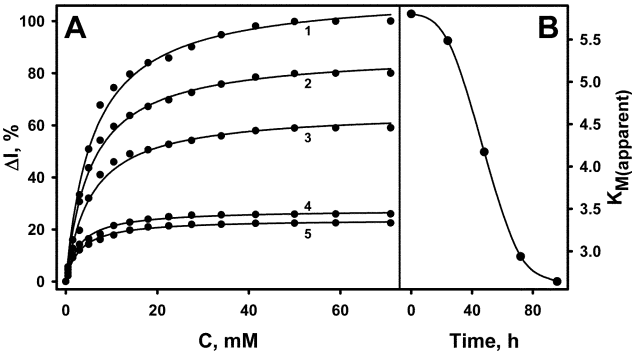

Fig. 5 (**A**) Glucose calibration curves obtained with a GOx-modified carbon electrode after different incubation periods in control solution 2 containing 300 mM of pyrrole (curves 1, 0; 2, 24 h; 3, 48 h; 4, 72 h; and 5, 96 h). (**B**) Calculated $K_{M(app)}$ *vs.* duration of incubation in 300 mM pyrrole solution. Amperometric detection was performed at +800 mV *vs.* Ag/AgCl in 50 mM sodium acetate buffer with 100 mM KCl, pH 6.0.

faster. Similar results were achieved when the bare buffer solution, without any pyrrole or glucose, was used for the same purpose (data not shown). After 96 h, the detected $K_{M(app)}$ was 2.6 which is close to K_M of native GOx; this means that the GOx layer became so thin that the sensor started to act in a kinetically-controlled mode instead of the previously detected diffusion-controlled mode.

The GOx-modified electrode exhibited an optimum pH of 6.5 (Fig. 6, curves 1 and 3), the same pH region that is characteristic for native GOx. A pH optimum was detected at pH = 7.2–7.4 (Fig. 6, curves 2 and 4) for the GOx/Ppy-modified electrode. The shift of 0.5–0.7 pH units was detected because of the influence of the Ppy matrix.

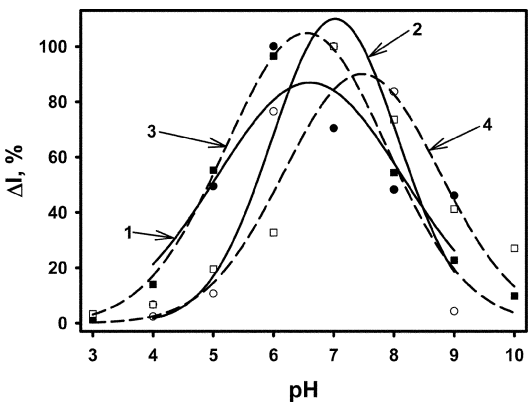

Fig. 6 Amperometric signal *vs.* pH of solution: (1) GOx-modified carbon electrode in 50 mM of potassium phosphate buffer (●); (2) GOx/Ppy-modified carbon electrode in 50 mM of potassium phosphate buffer (○); (3) GOx-modified carbon electrode in 25 mM of sodium acetate and 25 mM of potassium phosphate buffer (■); and (4) GOx/Ppy-modified carbon electrode in 25 mM of sodium acetate and 25 mM of potassium phosphate buffer (□). For each measurement, 30 mM of glucose added to the buffer containing 100 mM of KCl and 10 mM of PMS. Amperometric detection was performed at +300 mV *vs.* Ag/AgCl.

The stability of the GOx- and GOx/Ppy-modified electrodes was tested at 4 °C. It was detected that the GOx/Ppy-modified electrode was more stable for the first 30 days (Fig. 7A). After a

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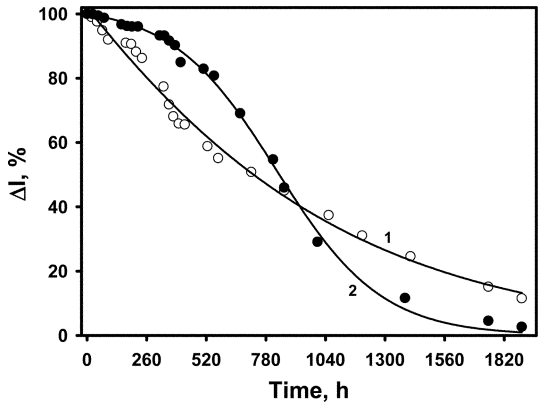

Fig. 7 Electrochemical signal *vs.* time: (1) GOx-modified carbon electrode, (2) GOx/Ppy-modified carbon electrode. Experiments were performed at +300 mV *vs.* Ag/AgCl in 50 mM of sodium acetate buffer with 100 mM of KCl and 10 mM of PMS, pH 6.0. 30 mM of glucose was added for each measurement.

30 day period a significantly reduced stability of the GOx/Ppy-modified electrode was detected. This should be taken into account in the development of these types of biosensor. Such a stability phenomenon is usual for enzymatic systems based on the application of diffusion barriers including polymeric layers. In the initial phase (if the enzyme is still very active), slow diffusion does not allow reaching of the maximal enzymatic reaction rate; however, after significant inactivation of the enzyme the role of diffusion becomes less important.

Conclusions

The new method proposed in this study is based on the self-encapsulation of enzymes within a polymeric layer, and opens up a new avenue for biosensor design. This method might be useful for the extension of the linear detection range of enzymatic biosensors based on oxidases and has several clear advantages compared with very common applications of diffusion membranes and the deposition of additional polymeric layers. The currently proposed method allows achievement of the required $K_{M(app)}$ and has some advantages compared with electrochemical deposition of the GOx/Ppy layer:[46] the equipment needed for the electrochemical polymerization is not required, and the Ppy deposition reaction can be terminated, by removal of the polymerization solution, once the required $K_{M(app)}$ is achieved or the polymerization reaction can be continued until the required $K_{M(app)}$ is reached. In addition, stabilization of the Ppy-modified enzymatic electrode is an important advantage of the proposed immobilization technique. In this study it is assumed that the proposed mild conditions for the self-encapsulation of immobilized enzyme are well suited for similar modification of biosensors based on other oxidases. It is supposed that such a technique may also be applied in the design of some enzymatic biofuel cells, because: (i) Ppy offers real biocompatibility and does not induce an immune response,[32] which will be required if biofuel cells are to be applied in self-powered biosensors or other bioelectronic devices that can be applied *in vivo*; (ii) Ppy is capable of transferring the charge generated by some redox enzymes directly,[21] which has potential application in fuel cells;[2] (iii) significant diffusion limitations within enzymatically formed Ppy[41] guarantees that secondary-substrates can be retained within the biocatalytic layer if consecutively acting enzymes[2,42] are applied in biofuel cell design;[2] and (iv) the Ppy layer prevents enzymes from leaching out of the biocatalytic layer.

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