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Unsubstituted phenothiazine as a superior water-insoluble mediator for oxidases

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ABSTRACT

The mediation of oxidases glucose oxidase (GOx), lactate oxidase (LOx) and cholesterol oxidase (ChOx) by a new electron shuttling mediator, unsubstituted phenothiazine (PTZ), was studied. Cyclic voltammetry and rotating-disk electrode measurements in nonaqueous media were used to determine the diffusion characteristics of the mediator and the kinetics of its reaction with GOx, giving a second-order rate constant of 7.6×10^3–2.1×10^4 M^{-1} s^{-1} for water–acetonitrile solutions containing 5–15% water. These values are in the range reported for commonly used azine-type mediators, indicating that PTZ is able to function as an efficient mediator. PTZ and GOx, LOx and ChOx were successfully co-immobilised in sol–gel membrane on a screen-printed electrode to construct glucose, lactate and cholesterol biosensors, respectively, which were then optimised in terms of stability and sensitivity. The electrocatalytic oxidation responses showed a dependence on substrate concentration ranging from 0.6 to 32 mM for glucose, from 19 to 565 mM for lactate and from 0.015 to 1.0 mM for cholesterol detection. Oxidation of substrates on the surface of electrodes modified with PTZ and enzyme membrane was investigated with double-step chronoamperometry and the results showed that the PTZ displays excellent electrochemical catalytic activities even when immobilised on the surface of the electrode.

1. Introduction

Amperometric biosensors based on oxidases have been described for over 80 analytes (May, 1999; Solná et al., 2005). Oxidases have evolved to catalyse the oxidation of many different substrates and are integral to the biological pathways of plants, animals, and bacteria. Among them the flavin adenine dinucleotide (FAD) dependent GOx (Cash and Clark, 2010; Li et al., 2011; Shan et al., 2010) and cholesterol oxidase (ChOx) (Arya et al., 2008; Fang et al., 2011) as well as flavin mononucleotide (FMN) dependent lactate oxidase (LOx) (Pereira et al., 2011; Romero et al., 2010) are suitable candidates for the recognition of important physiological analytes. The use of mediators to create disposable amperometric biosensors for analytes such as glucose has proved to be a highly successful strategy and a major commercial success (Newman and Turner, 2005). Direct electron exchange between an electrode surface and the deeply buried prosthetic group of large enzymes such as glucose oxidase (160 kDa) is hampered (Wilson and Turner, 1992). Electron transfer mediators facilitate electrical communication between the active site of a redox protein and the electrode thus decreasing the kinetic barrier for electron transfer (Cui et al., 2007; Liu et al., 2009).

A typical electrocatalytic reaction for a mediated oxidase proceeds in three steps. The enzyme takes part in first redox reaction with the substrate and is then re-oxidised by the mediator. Finally the mediator is re-oxidised by the electrode. In this context, a variety of reversible electron acceptors have been studied as mediators for redox enzymes. The electron transfer mediator has to satisfy the following requirements: (i) the redox potential of the mediator should be small enough to avoid interfering electrochemical reactions, (ii) both oxidised and reduced forms of mediator should be stable enough and (iii) the second-order rate constant for reaction between mediator and enzyme should be high enough to minimise competition with oxygen (Cardosi and Turner, 1987). Consequently, the choice of the mediator is critical to achieve high sensitivity and selectivity.

Ferrocene and its derivatives, ferri/ferrocyanide, complexes of transition metals such as osmium and ruthenium as well as redox organic dyes are widely used redox mediators for oxidases. Ferri/ferrocyanide is one of the most commonly used and efficient soluble inorganic mediators (Dubinin et al., 1991; Jaffari and Turner, 1997; Shul'ga et al., 1994). However due to its small size

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and high solubility, it easily diffuses away from the electrode surface into the bulk solution, which reduces long-term operational stability and hampers its application in both continuous laboratory analysers and implantable probes. The less soluble ferrocene derivatives provide a partial solution to this problem and have achieved considerable success in commercial home-use devices, but the ferrocinium ion is still soluble (Cass et al., 1984). Tertrathiafulvalene has been proposed as one alternative to ferrocene derivatives as an insoluble mediator for amperometric biosensors (Palleschi and Turner, 1990); (Turner et al., 1987); (Murthy and Anita 1996). In spite of their high efficiency in mediating the electron transfer of oxidases, osmium and ruthenium complexes (Motonaka et al., 1994; Ohara et al., 1993) have essential drawbacks due to their high toxicity. Several redox dyes such as methylene green (Upadhyay et al., 2009), methylene blue (Wang and Hasebe, 2012), meldola blue (Pereira et al., 2011), celestine blue (Noorbakhsh et al., 2008), phenazine (Liu et al., 1997; Ohfuji et al., 2004), thionine (Huang et al., 2011), azure B (Shan et al., 2003), and toluidine blue (Yao and Shiu, 2007) can be used as electron transfer mediators for oxidases when immobilised on the electrode surfaces. The main drawback of organic dyes-based biosensors is the leaching out of catalytic materials from the electrode surface. Thus many studies have been done to improve the operational stability of such devices. In our previous work (Sekretaryova et al., 2012) we have evaluated the possibility to eliminate mediator leakage by incorporating a new hydrophobic mediator, unsubstituted phenothiazine (PTZ), within a robust water-insoluble sol–gel membrane of siloxanes.

In the present study, the protocol for enzyme co-immobilisation with PTZ into a siloxane sol–gel membrane has been expanded to different oxidases. Kinetic studies of PTZ-mediated bioelectrocatalysis by a variety of methods demonstrated the excellent operational performance of the mediator as an electron shuttle between the active site of these various oxidases and the electrode. The entrapment of PTZ into a siloxane sol–gel matrix led to the development of a robust and flexible environment for immobilisation of different oxidases: GOx, LOx and ChOx. A reagentless biosensor based on mediated ChOx has been further characterised to show superior analytical performance compared to other biosensors for cholesterol measurement.

2. Experimental

2.1. Materials

All inorganic salts, organic solvents and Triton X-100 were obtained at the highest purity from Sigma Aldrich (Sweden). γ-Aminopropyltriethoxysiloxane was obtained from Reachim (Moscow, Russia). Glucose oxidase type VII from *Aspergillus niger* (lyophilised powder, ≥ 100 U mg^{-1} solid), cholesterol oxidase from *Streptomyces sp*. (lyophilised powder, ≥ 20 U mg^{-1} protein), cholesterol (powder, BioReagent, suitable for cell culture, $\geq 99.0\%$), lactate oxidase from *Pediococcus sp.* (lyophilised powder, ≥ 20 U mg^{-1} solid), Sodium L-lactate ($\geq 99.0\%$ (NT)) were purchased from Sigma Aldrich (Sweden). D-Glucose was obtained from AnalaR (England). All chemicals were of reagent grade and used as received. Experiments were carried out with Milli-Q water from a Millipore Milli-Qsystem.

An aqueous stock solution of 0.5 M glucose was prepared in phosphate buffer (0.05 M, pH 7.0) and left for at least 24 h at room temperature before use to allow equilibration of the anomers. The solution was stored at 4 °C.

A stock solution of 15 mM cholesterol was prepared as previously described (Vidal et al., 2004) in phosphate buffer (0.05 M, pH 6.8) containing 15% (w/w) of Triton X-100 in a thermostated bath at 65 °C. This solution was stored at 4 °C in the dark and was stable for at least 10–15 days (until turbidity was observed). More dilute working solutions of cholesterol were prepared by dilution of the stock solution using a 0.05 M phosphate buffer solution containing 1% (w/w) of Triton X-100.

A stock solution of 4 M lactate was prepared by dissolving the proper amount of sodium lactate in phosphate buffer (0.05 M) solution pH 6.0. The solution was stored at 4 °C.

2.2. Apparatus

An Autolab type III bipotentiostat system (Autolab, EcoChemie, Netherlands) and EmStat USB potentiostat (Palm Instruments, Netherlands) were employed for cyclic voltammetry (CV) and chronoamperometric measurements. The screen-printed electrode system with graphite working electrode (diameter 1.9 mm), graphite auxiliary electrode, and Ag/AgCl reference electrode were purchased from Rusens Ltd. (Moscow, Russia) and were characterised by reversible voltammograms of ferro/ferrycyanoferrate couple in neutral media. Rotating disk electrode (RDE) voltammetry was performed using a potentiostat supplied by Ivium Technologies (USA) using a conventional three-electrode cell configuration. Glassy carbon (GC) rotating disk electrode (diameter 5 mm, Pine Instrument) was used as the working electrode with rotation control (Pine Instrument).

2.3. Measurements in acetonitrile

GC electrodes were successively polished with alumina powders (1.0 and 0.05 μm) followed by ultrasonication in distilled water and washing with ethanol. Measurements were carried out in 0.1 M $LiClO_4$ acetonitrile solution containing 1 mM PTZ. A platinum wire and an Ag+/Ag (0.01 M $AgNO_3$, 0.1 M $TBAClO_4$ in acetonitrile) electrode were used as the auxiliary and reference electrodes for all measurements in acetonitrile. Cyclic voltammogram of ferrocene (1 mM in acetonitrile) has been recorded on glassy carbon electrode as a test experiment to check the validity of organic reference electrode. Glucose oxidase and glucose aqueous solutions were added into the cell. Final water contents in CV and RDE measurements were 15% and 5% respectively.

2.4. Enzyme immobilisation

Enzyme immobilisation was carried out from water–organic mixture with high content of organic solvent (Yashina et al., 2010). Aqueous solution of the appropriate enzyme (GOx or ChOx or LOx) was suspended in an isopropanol solution of γ-aminopropyltriethoxysiloxane containing PTZ. 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). The biosensors response was assayed in 0.05 M phosphate buffer solutions containing 0.1 M KCl as a supporting electrolyte. The pH of the buffer solutions were selected to be appropriate for the enzymes used (Table 1).

3. Results and discussion

Presumably because of its insolubility in water, PTZ has not been previously explored as a mediator for oxidases. However, in order to ensure the electron transfer, the mediator must be present in both oxidised and reduced forms, which must remain in the vicinity of the electrode. In some formats, this means that the mediator must be insoluble. Hence, this is the first detailed