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Amperometric glucose biosensor based on layer-by-layer films of microperoxidase-11 and liposome-encapsulated glucose oxidase

J.S. Graça [a], R.F. de Oliveira [b], M.L. de Moraes [c], M. Ferreira [a,*]

[a] Federal University of São Carlos, UFSCar, Campus Sorocaba, 18052-780 Sorocaba, SP, Brazil
[b] São Paulo State University, UNESP, Postgraduate Program in Materials Science and Technology, 17033-360 Bauru, SP, Brazil
[c] Federal University of São Paulo, UNIFESP, Campus São José dos Campos, 12231-280 São José dos Campos, SP, Brazil

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ABSTRACT

An important step in several bioanalytical applications is the immobilization of biomolecules. Accordingly, this procedure must be carefully chosen to preserve their biological structure and fully explore their properties. For this purpose, we combined the versatility of the layer-by-layer (LbL) method for the immobilization of biomolecules with the protective behavior of liposome-encapsulated systems to fabricate a novel amperometric glucose biosensor. To obtain the biosensing unit, an LbL film of the H_2O_2 catalyst polypeptide microperoxidase-11 (MP-11) was assembled onto an indium-tin oxide (ITO) electrode followed by the deposition of a liposome-encapsulated glucose oxidase (GOx) layer. The biosensor response toward glucose detection showed a sensitivity of 0.91 ± 0.09 (μA/cm^2)/mM and a limit of detection (LOD) of 8.6 ± 1.1 μM, demonstrating an improved performance compared to similar biosensors with a single phospholipid-liposome or even containing a non-encapsulated GOx layer. Finally, glucose detection was also performed in a zero-lactose milk sample to demonstrate the potential of the biosensor for food analysis.

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1. Introduction

Preserving the activity of immobilized enzymes is a crucial factor for developing successful biosensors based on enzyme catalysis [1–3]. Thus, several strategies, such as physical adsorption, covalent binding, entrapment and cross-linking, have been explored to retain the enzyme native structure onto various types of electrodes [4–6]. These immobilization protocols may affect biosensor efficiency in terms of operational and storage stabilities, sensitivity, selectivity, response time and reproducibility [7], which is related to the enzyme orientation, loading, mobility, stability, structure and biological activity during and after immobilization. For this reason, intensive efforts have been made to employ a successful immobilization strategy that ensures an improved biosensor performance [2]. The layer-by-layer technique (LbL), a widely explored adsorption method, has been an effective approach for enzyme immobilization due to its simplicity and the mild conditions during the film formation [8,9]. Because most enzymes are water-soluble and charged in solution, the LbL method is suitable for assembling different enzyme architectures with low denaturing effects [10,11]. The LbL method can be basically described as a deposition procedure for immobilization of oppositely charged species such as polymers, proteins and nanoparticles, from aqueous solutions [11]. Although many different forces may be involved in film formation, most LbL assemblies are driven by electrostatics [11]. Due to its unique characteristics, such as its applicability to a wide variety of materials, low-cost and biocompatibility, the LbL method has been extensively used in many applications. These include light-emitting diodes (LEDs) [12], solar cells [13], and field-effect transistors [14] as antibacterial [14] and antireflective [15] coatings and many other applications [16].

In biosensing, LbL films have been used in various ways. Ferreira et al., for example, reported a successful strategy for glucose detection using LbL films of glucose oxidase (GOx) and polyallylamine hydrochloride (PAH) on modified electrodes [17]. Lee et al. developed a label-free immunoassay method using an LbL network of carbon nanotubes for detecting swine influenza H1N1virus [18]. Xiang et al. assembled multi-enzyme LbL films of carbon nanotubes for the electrochemical monitoring of important cancer biomarkers [19]. An overview of LbL applications in biosensing and related fields has recently been published [11]. In addition to the LbL strategy, liposome encapsulation has been exploited to preserve the native structure of biomolecules for biosensing [20,21]. The enclosed structure of a liposome shell isolates the encapsulated biomolecule from possible environmental stresses caused by unsuitable pH, ionic strength and temperature [20,21]. Yoshimoto et al., for instance, obtained higher storage and thermal stabilities for yeast alcohol dehydrogenase encapsulated in liposomes [22]. Petri et al. used LbL films to achieve an improved biosensor response when HIV p17-1 peptide was protected by liposomes [20]. The secondary

* Corresponding author at: UFSCar, Rodovia João Leme dos Santos, Km 110 - SP - 264, Bairro do Itinga, Sorocaba, SP, Brazil. Tel.: +55 15 3229 5969.
E-mail addresses: ju_graca@yahoo.com.br (J.S. Graça), furlanrafa@hotmail.com.br (R.F. de Oliveira), marli.moraes@gmail.com (M.L. de Moraes), marystela@ufscar.br (M. Ferreira).

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structure of the peptide incorporated into liposomes was preserved, but the same did not apply to LbL films with unprotected p17-1. Liu et al. [23] recently reviewed the use of liposomes in biosensors.

In this work, the benefits of liposome encapsulation for preserving the native structure of proteins were investigated in the response of LbL-based glucose biosensors. We exploited the H_2O_2 catalytic properties of microperoxidase-11 (MP-11) as an electron mediator element. MP-11 was chosen due to its relatively simple structure and the similarity of its catalytic behavior to that of peroxidase enzymes. Single phospholipid-liposomes and a mixture of two phospholipids were tested for GOx encapsulation. The biosensor containing MP-11 and a liposome-encapsulated GOx layer was used to detect glucose in zero-lactose samples to demonstrate its potential in food analysis.

2. Experimental details

2.1. Materials

The microperoxidase-11(MP-11),disodium salt from equine heart cytochrome c, 1925 g/mol, with an isoelectric point at pH 4.75 [24] and optimal activity between pH 4.5 and 8.0 [24] was purchased from Sigma Aldrich. Glucose oxidase (GOx) from *Aspergillius niger* (E.C. 1.1.3.4), 118 units/mg, with an isoelectric point at pH 4.2 [25], pKa of 8.2 [25] and optimal activity between pH 3.5 and 6.5 [25], was purchased from Fluka. Poly(ethylene imine) (PEI) and the phospholipids 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol(POPG) and 1,2-dipalmitoyl-phosphatidylglycerol (DPPG) were purchased from Sigma-Aldrich and Avanti Polar Lipids, respectively. Glucose (Sigma-Aldrich) solutions used to determine the biosensor response were allowed to mutarotate overnight. All other reagents were of analytical grade and used without further purification.

2.2. Methods and instrumentation

The LbL films were assembled onto quartz and ITO-coated glass substrates (indium-tin oxide, one side coated on glass by Delta Technologies) for spectroscopic and electrochemical measurements, respectively. The quartz substrates were previously cleaned in $HCl/H_2O_2/H_2O$ (1:1:6) (v/v) and $NH_4OH/H_2O_2/H_2O$ (1:1:5) (v/v) solutions, both for 10 min at 80 °C. ITO substrates were cleaned using chloroform, isopropanol and ultrapure water (Milli-Q).

Solutions of MP-11 (0.15 mg/mL), PEI (1 mg/mL), GOx and liposome-encapsulated GOx (both 1 mg/mL) were all prepared in phosphate buffer solution (PBS) at 10 mM and pH 6.3. For spectroscopic measurements, a 2-bilayer precursor LbL film of PEI/PVS (polyvinylsulfonic acid) was assembled onto quartz to reduce the influence of the substrate morphology on film growth [20]. Afterwards, 8-bilayer LbL films were produced by alternately dipping the substrate into the polycationic solution (PEI) for 3 min and the anionic solution (MP-11) or liposome-encapsulated GOx for 10 or 5 min, respectively, with a rinse step in PBS for 30 s in between. The multilayer buildup was monitored at each deposition step using a Genesys 6 UV–visible spectrophotometer (Thermo Fischer). The growth curves were plotted using the average values of absorbance obtained from three different experiments. The solutions of liposome-encapsulated GOx were prepared as described elsewhere [26]. Here, GOx was encapsulated into a mixture of DPPG + POPG (1:4) (v/v) liposomes. This ratio was chosen after an optimization study (results not shown). A single bilayer of GOx or (GOx + POPG + DPPG) was assembled on a 2-bilayer PEI/MP-11 film on ITO to produce two biosensing units. For this purpose we employed deposition times of 3 min in PEI solution, and 30 min in GOx or liposome-encapsulated GOx solutions.

Circular dichroism (CD) spectroscopy was used to analyze the GOx structure in solution (both free and incorporated into the liposome mixture) and also as an LbL film. CD spectra in PBS were collected using a quartz cell of 1 mm optical path. The CD spectra of the 10-bilayer PEI/GOx and PEI/liposome-encapsulated GOx LbL films were performed on quartz substrates. In this case, the optical path was given by the film thickness. Measurements were performed on a J-815 CD spectrometer (Jasco Inc.), with a band width of 1 nm, a response time of 0.5 s and a scanning speed of 100 nm/min. CD spectra were obtained by averaging eight scans.

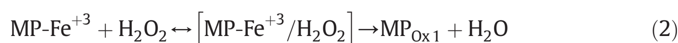
Amperometric measurements were carried out in a 3-electrode electrochemical cell (7 mL) at room temperature using an Autolab PGSTAT 30 (Echochemie). A platinum sheet (1 cm^2) was used as the counter electrode and the LbL-modified ITO substrates were used as the working electrodes. All potentials were referred to a saturated calomel electrode (SCE). All electrochemical data were acquired in PBS (10 mM, pH 6.3) under stirring and at 50 mV [27]. Glucose and H_2O_2 solutions were also prepared in PBS for determining the biosensor response and MP-11 activity, respectively. All amperometric experiments were performed three times, and the sensitivity and limit of detection (LOD) values were obtained as an average. Before all amperometric experiments the potential of each biosensor was held at the operating value, allowing the background current to decay to a steady-state value.

3. Results and discussion

Scheme 1 shows the idealized structure of the glucose biosensor. A 2-bilayer LbL film of PEI/MP-11 was assembled onto the ITO electrode, followed by the deposition of a single bilayer of PEI/liposome-encapsulated GOx.

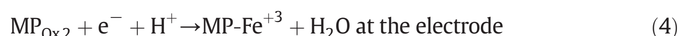
The detection mechanism relies on the cascade reaction shown by Eqs. (1) to (4). The initial catalysis of β-D-glucose by GOx produces H_2O_2, which is subjected to MP-11 catalysis and produce the electrical signal [28].

$$\beta\text{-D Glucose} + O_2 \xrightarrow{GOx} \text{Gluconic acid} + H_2O_2 \quad (1)$$



$$MP\text{-}Fe^{+3} + H_2O_2 \leftrightarrow \left[MP\text{-}Fe^{+3}/H_2O_2\right] \rightarrow MP_{Ox\,1} + H_2O \quad (2)$$

$$MP_{Ox\,1} + e^- + H^+ \rightarrow MP_{Ox\,2} \quad (3)$$



$$MP_{Ox\,2} + e^- + H^+ \rightarrow MP\text{-}Fe^{+3} + H_2O \text{ at the electrode} \quad (4)$$

where MP_{Ox1} and MP_{Ox2} are two intermediate forms of MP-11[29].

3.1. PEI/MP-11 LbL film growth and activity

Fig. 1 shows the UV–vis absorption spectra for the 8-bilayer PEI/MP-11 LbL film. The absorbance increase at 413 nm was monitored for each deposited layer (inset in Fig. 1). The pronounced band at approximately 413 nm in the film spectra can be assigned as the Soret band, which is assigned to the $\pi \rightarrow \pi^*$ transition in the porphyrin cycle of the heme group of MP-11 [30,31]. Less intense bands at ca. 354 and 530 nm can be attributed to the N and Q bands, respectively [30,31]. The Soret band in Fig. 1 is red shifted, from 400 to 413 nm, compared to the MP-11 solution spectrum (not shown). This shift is consistent with a more non-planar conformation of the porphyrin macrocycle [32]. Astuti et al. observed a red shift of the Soret band from 400 to 409 nm when MP-11 was removed from PBS and immobilized onto a SnO_2 electrode using the polycationic binder poly-L-lysine [33]. Huang et al. observed a 10 nm red shift when MP-11 was immobilized in cationic lipid vesicles, but no band shift occurred when neutral lipids were used [32]. A subsidiary experiment (not shown) was performed by titrating PEI droplets into the MP-11 solution, in a manner similar to that reported by Astuti et al. using also PEI and DDAB (dodecyldimethylammonium bromide) solutions [33]. In both cases, an 8 to 10 nm red shift was observed. All of these results are consistent with a slight change in the MP-11 microenvironment due to the presence of cationic species, similar to the PEI/MP-11 LbL films reported here. This film also shows a

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