Materials Science and Engineering C 58 (2016) 310–315

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Monoamine oxidase B layer-by-layer film fabrication and characterization toward dopamine detection

Celina Massumi Miyazaki [a], Tamyris Paschoal Pereira [a], Daniela Branco Tavares Mascagni [b], Marli Leite de Moraes [c], Marystela Ferreira [a,*]

[a] Universidade Federal de São Carlos, UFSCar, CCTS, Sorocaba, São Paulo, Brazil
[b] Universidade Estadual de São Paulo — UNESP, Sorocaba, São Paulo, Brazil
[c] Universidade Federal de São Paulo, Unifesp, São José dos Campos, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 17 June 2015
Received in revised form 13 August 2015
Accepted 22 August 2015
Available online 29 August 2015

Keywords:
Layer-by-layer
Monoamine oxidase-B
Dopamine detection
Liposomes
Amperometry

ABSTRACT

In this work nanostructured film composites of the monoamine oxidase B (MAO-B) enzyme, free or encapsulated in liposomes, were fabricated by the layer-by-layer (LbL) self-assembly technique, employing polyethylene imine (PEI) as polycation. Initially, the MAO-B enzyme was incorporated into liposomes in order to preserve its enzymatic structure ensuring their activity and catalytic stability. The LbL film growth was monitored by surface plasmon resonance (SPR) by gold resonance angle shift analysis after each bilayer deposition. Subsequently, the films were applied as amperometric biosensors for dopamine detection using Prussian Blue (PB) as the electron mediator. The biosensor fabricated by MAO-B incorporated into liposomes composed of DPPG:POPG in the ratio (1:4) (*w*/*w*) showed the best performance with a sensitivity of 0.86 ($\mu A\ cm^{-2}$)/($mmol\ L^{-1}$) and a detection limit of 0.33 $mmol\ L^{-1}$.

1. Introduction

Nowadays, many researches are focused on the development of new sensors with faster and sensitive diagnosis in many different areas, such as biomedical, industrial and environmental monitoring, aiming to improve the welfare and health quality. Several methods have been applied, but up to date the use of electrochemical methods with modified electrodes for sensor and biosensor applications has provided the easiness of combining fast response, easy operation, selectivity and sensitivity in low cost chemical analysis methods [1,2]. Various materials have been used in the electrode modifications toward selectivity and sensitivity enhancement, such as graphene [3,4], nanotubes [5–7], nanoparticles [8,9], conducting polymers [10,11], enzymes [12–15], etc.

Biosensors can be constructed by several methodologies, whereas the layer-by-layer (LbL) technique developed in the 90s by Decher et al. [16] has been widely used in the development of electrochemical biosensors [13,14,17] by allowing the use of the synergistic properties of different materials in a simple and versatile way, and to facilitate the control of manufacturing conditions in order to preserve the biomolecules' activity [18]. This technique allows for the immobilization of various kinds of materials such as charged biomolecules that include: DNA, enzymes, proteins and phospholipids, all using basic equipment at a relatively low cost.

Biosensors based on the immobilization of enzymes require special care to maintain their natural conformation and activity. The incorporation of liposomes is an alternative for the conservation of the biomaterial properties improving the sensitivity and the detection limit of the device [13,14,19,20].

Monoamine oxidase B (MAO-B) is a protein localized in the outer membrane of the mitochondria that catalyzes the oxidation of amines, such as dopamine and tyramine [21]. Dopamine (DA) is an important neurotransmitter of a class of molecules called catecholamine. Inadequate amounts of dopamine in our brain can lead to the development of diseases such as Parkinson's and schizophrenia [22], therefore, its quantification is extremely important for the diagnosis and monitoring of these diseases. Because of the relation of DA concentration to the occurrence of various diseases, the development of sensors for its quantification has attracted attention in recent years. Several analytical techniques have been developed for the DA determination including sophisticated methods such as mass spectrometry [23], optical spectroscopy [24–26], high performance liquid chromatography (HPLC) [27,28], and more simple and cheaper electrochemical methods [3,29–35]. Electrochemical techniques are the most exploited for DA detection, however, its quantification becomes complicated due to the presence of natural interferents such as ascorbic (AA) and uric (UA) acid, which have similar oxidation potentials to the DA [22]. The differential pulse voltammetry method applying materials capable of

* Corresponding author at: Rod. João Leme dos Santos, Km 110, CEP 18052-780 Sorocaba, São Paulo,Brazil.
E-mail address: marystela@ufscar.br (M. Ferreira).

http://dx.doi.org/10.1016/j.msec.2015.08.042
0928-4931/

promoting the separation of the oxidation peaks of the different analytes (DA, AA and UA) has been the center of the research for DA sensors [3,32,34–36]. Here we propose an amperometric sensor using the MAO-B enzyme for specific detection of DA using PB as a mediator which allows for low potential measurements and avoids an interfering response. A range of materials has been used for the development of these devices, such as nanoparticles [33,35,37], phthalocyanines [38,39], carbon nanotubes [40,41] and graphene derivatives [3,33,36], among others.

In this study the MAO-B was immobilized in LbL films in two different ways, free or incorporated in liposomes, using polyethylene imine (PEI) as polycation. The biosensor was applied as an amperometric biosensor using Prussian Blue (PB) as an electron mediator [42]. Sensitivity, detection limit, stability and interferent tests were performed to analyze the biosensors.

2. Experimental details

2.1. Materials

Monoamine oxidase B human (MAO-B) with an isoelectric point of 5.3 and with optimal activity between pH 7.0 and 7.4, was purchased from Sigma-Aldrich. The phospholipids Dipalmitoyl phosphatidyl glycerol (DPPG) and palmitoyl phosphatidyl gliceral (POPG) were obtained from Sigma-Aldrich and Avanti Polar Lipids, respectively. The polyelectrolytes poly (ethyleneimine) (PEI), poly (vinyl sodium sulfate) (PVS) and dopamine hydrochloride (DA) were obtained from Sigma-Aldrich. The reagents were used without treatment. Solutions were prepared in a pH 7.4 0.1 mol L^{-1} potassium phosphate buffer (PBS).

2.2. MAO-B incorporation in liposomes

Liposomes were prepared as references [13,43] using a mixture of POPG and DPPG phospholipids 1:4 (*w*/*w*) (chosen after an optimization study, not shown). Briefly, phospholipids were solubilized in a chloroform:methanol mixture 8:2 (*v*/*v*) and evaporated in a rotary evaporator (IKA RV 10, HB 10, USA) at 36 °C. Then, MAO-B solution (0.2 mg mL^{-1}) was added to the lipid film and left in an ultrasound bath for 2 h for the enzyme's incorporation into the liposomes. The MAO-B solutions were analyzed by circular dichroism (CD) spectroscopy with the J-815 CD spectrometer equipment (Jasco Inc., USA).

2.3. Prussian Blue (PB) film electrodeposition onto the ITO substrates

For the electrochemical measurements ITO (tin oxide doped with indium, Delta Technologies) substrates were applied after cleaning with chloroform and isopropilic alcohol. ITO slides were modified by electrode deposition of the Prussian Blue (PB) applying a potential of +0.40 V for 100 s in a mixture of 5.0 mL of 2.0 mmol L^{-1} $K_3Fe(CN)_6$ solubilized in KCl (100 mmol L^{-1}) and 5.0 mL of 2.0 mmol L^{-1} $FeCl_3$ solubilized in HCl (10 mmol L^{-1}) [17,44]. The modified electrode was washed with ultrapure water and immersed in 100 mmol L^{-1} KCl and 10 mmol L^{-1} HCl, then an ITO/PB electrode was cycled from 0.0 to 1.0 V (vs SCE) at a scan rate of 0.05 V/s, until the voltammetric behavior stabilized.

2.4. LbL film assembly

All solutions for LbL assembly were prepared with 0.1 mol L^{-1} PBS solution pH 7.4, previously prepared from ultrapure water (18.2 MΩ cm at 25°°C). Before receiving the enzyme film, two layers of PEI/PVS were deposited to reduce the influence of surface irregularities [45]. 1.0 mg mL^{-1} PEI solution and 1.3 mg mL^{-1} PVS solution and an immersion time of 3 min for both polyelectrolytes were used. For PEI/MAO-B film assembly, we immersed the ITO/PB in a PEI solution (1 mg mL^{-1}, 3 min) and alternated by immersing in a MAO-B solution (0.2 mg mL^{-1}, either free or incorporated in liposomes, 10 min). Each deposition was followed by a washing solution (PBS) for 30 s to remove any weakly absorbed material. To analyze the LbL film growth, the SPR Navi 200 surface plasmon resonance analyzer (BioNavis, Finland) was used, with a 670 nm laser and thiol functionalized gold surface (12 h in a 150 mmol L^{-1} 11-mercaptoundecanoic acid ethanolic solution).

2.5. Electrochemical measurements

The amperometric measurements were performed with an Autolab PGSTAT 30 galvanostat/potentiostat using an electrochemical cell that consisted of a saturated calomel reference electrode (SCE), a platinum foil as an auxiliary electrode and the working electrode containing ITO/PB recovered with the free enzyme film $(PEI/(MAO\text{-}B)_7$ and liposome incorporated enzyme film $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)_7$ at a potential of 0.0 V. PBS was used as support solution.

3. Results and discussions

3.1. Structural analysis of MAO-B free and incorporated in liposomes in solutions

Studies with circular dichroism (CD) give an indication of the secondary structure of proteins. Fig. 1 illustrates the CD spectra for free MAO-B and incorporated in liposomes 1POPG:4DPPG solutions in PBS. The spectrum of MAO-B incorporated in liposomes indicated two minimums of 208 nm and 223 nm and a positive band at 197 nm with a negative to positive crossover at 201 nm, characteristic of an α-helix structure [46,47]. The spectrum of the free MAO-B in the buffer showed only a minimum at 207 nm, possibly due to conformational changes in the absence of the liposome.

3.2. LbL film growth

The monitoring of the LbL film growth was performed by surface plasmon resonance (SPR). Fig. 2A shows the spectra of each deposited bilayer. The minimum angle of reflection θ due to absorption of the plasmon by the thiol-modified gold surface was 68.689°. With the addition of each bilayer, θ presented a sequential increase. Fig. 2B shows the shift in SPR angle Δθ with the deposition of the bilayer. Since the magnitude of Δθ is dependent upon the thickness and the refractive index of the deposited layers on the gold surface [48], we can believe in a linear growth behavior on the LbL films. In the presence of liposomes Δθ was higher, a fact already predicted due to the fact that liposomes are relatively large vesicles (100–1000 nm) and cause, therefore, greater changes in the surface refractive index when attached to the surface [49].

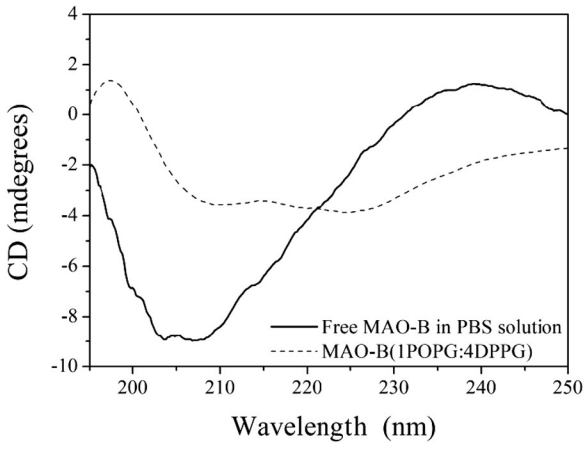

Fig. 1. CD spectra for free MAO-B and liposome incorporated MAO-B solutions in PBS.

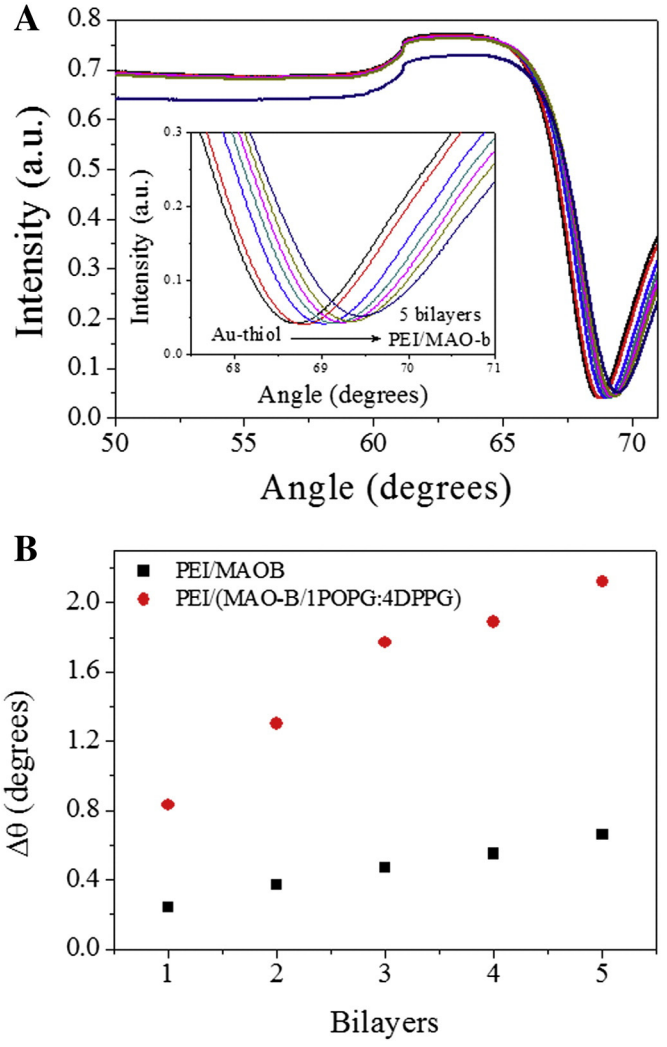

Fig. 2. (A) SPR curves for PEI/MAO-B bilayers onto a thiol-modified Au sensor. The number of deposited layers increased from left to right. (B) SPR angles shifted as a function of the deposited layers of $(PEI/MAO\text{-}B)_5$ e $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_5$.

3.3. Electrochemical response of the LbL films

Fig. 3 shows an idealized scheme for the operation of the designed biosensor containing the MAO-B encapsulated in liposomes as used for detecting dopamine. MAO-B catalyzes the oxidation of DA generating H_2O_2 as a byproduct, which is reduced by the mediator PB at low potential, this helps to eliminate possible electroactive interferents existing in real samples [50]. Eq. (1) describes the reduction of PB when forming the Prussian White (PW), with Fe^{III} to Fe^{II} representing the oxidation states of Fe [17].

$$KFe^{III}Fe^{II}(CN)_6\ (PB) + e- + K^+ \rightarrow K_2Fe^{II}Fe^{II}(CN)_6\ (PW) \quad (1)$$

For the amperometric experiments we used an electrolyte of 0.1 mol L^{-1} PBS pH 7.4 at a potential of 0.0 V [50]. Biosensors composed by ITO/PB coated with films $(PEI/MAO\text{-}B)_7$ and $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ were tested. Pure ITO and ITO/PB (in the absence of the films) showed no response to DA.

Amperometric responses for biosensors were shown in Figs. 4A and B, respectively for the $(PEI/MAO\text{-}B)_7$ and $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ films. Both films presented a fast response to the addition of DA solution about 45 s. The inset shows the calibration curve plotted. The linear regression equation for $(PEI/MAO\text{-}B)_7$ was j (μA) = $0.095C_{DA}$ (mmol L^{-1}) − 0.027 with a correlation coefficient of $R^2 = 0.952$. The detection limit (S/N = 3) calculated was 1.41 ± 0.06 mmol L^{-1}. However, for the film composed by $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ the linear regression equation was j (μA) = $0.304C_{DA}$ (mmol L^{-1}) + 2.020 with $R^2 = 0.996$ and the detection limit was 0.86 ± 0.16 mmol L^{-1}.

When the MAO-B enzyme was found incorporated in the liposomes, the sensitivity of the sensor was superior. Because of the similar structure to cell membranes, liposomes provide a biocompatible environment, promoting the stabilization of the enzyme conformation due to hydrophobic interactions between their structure and the lipid bilayers. Table 1 shows the sensitivity and detection limit of the tested biosensors. ITO/PB covered with $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ film presented a higher sensitivity and lower detection limit, which could be related to a strong combination in the structural formation of lipids by mixing one POPG:DPPG 1:4 (*w*/*w*) [13] thus leading to the preservation of the enzyme structure. Considering the temperature of the transition phase of the individual lipids ($T_m = -2$°C for POPG and $T_m = 41$°C for DPPG), the 1:4 mixture allows an ideal structure to immobilize MAO-B and provide the analyte penetration more effectively through lipid structures [13].

The apparent Michaelis–Menten constant (K_m^{app}) was calculated by the Lineweaver–Burk equation (double reciprocal), shown in Eq. (2) [51]. K_m^{app} provides an indication of enzyme-substrate kinetics and, consequently, it is related to the enzymatic activity.

$$\frac{1}{i_{ss}} = \frac{K_m^{app}}{i_{máx}} \times \frac{1}{C_{DA}} + \frac{1}{i_{máx}} \quad (2)$$

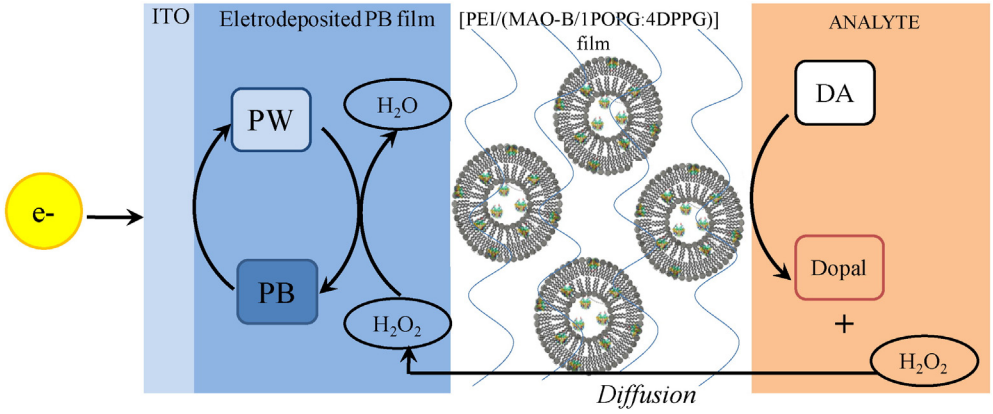

Fig. 3. Idealized mechanism of ITO/PB containing the MAO-B enzyme encapsulated in liposomes. DA is oxidized, which generates hydrogen peroxide which is transmitted by diffusion through the film and reduced by PB. PW: Prussian Write, PB: Prussian Blue, DA: dopamine.

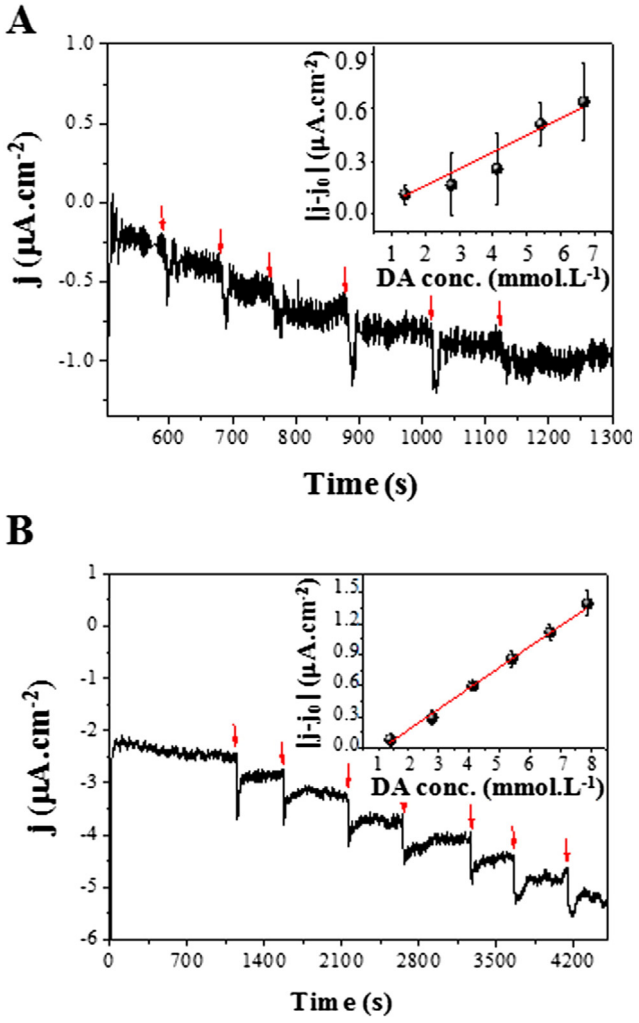

Fig. 4. Amperometric response obtained at E = 0.0 V in 0.1 mol L^{-1} PBS pH 7.4 to the ITO/PB covered with (A) $(PEI/MAO\text{-}B)_7$ film and (B) $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ film. Each current step indicates an increase of 100 μL of 0.1 mol L^{-1} DA solution.

where i_{ss} is a steady-state current, C_{DA} is the DA concentration, K_m^{app} is the apparent Michaelis–Menten constant and $i_{máx}$ is the maximum current [51]. K_m^{app} values were obtained from the slope and the intercept point for a plot of $1/i_s$ versus $1/C_{DA}$. The lower K_m^{app} was calculated for ITO/PB covered with $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$, indicating better enzymatic activity.

The stability of the films was tested by analyzing the analytical response for several days. After each test, biosensors were stored at 10 °C after rinsing with the PBS and drying. The biosensor composed by the $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ film presented no changes in the sensitivity over the first 3 days. After the 5th day the sensitivity decreased by 33% and after 10 days it had decreased by 56%. The sensor composed of the free enzyme $(PEI/MAO\text{-}B)_7$ presented a loss of sensitivity after its first use, probably due to the low stability of the enzyme in the free state.

Table 1
Sensitivity, detection limit, and apparent Michaelis–Menten constant (K_m^{app}) of the biosensors tested.

ITO/PB+	$(PEI/MAO\text{-}B)_7$	$[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$
Sensitivity (μA cm^{-2})/(mmol L^{-1})	0.940 ± 0.001	0.33 ± 0.02
Detection limit (mmol L^{-1})	1.41 ± 0.07	0.86 ± 0.16
K_m^{app} (mmol L^{-1})	13.53 ± 5.29	6.59 ± 0.82

The interfering effects in the biosensor's responses were investigated to assess its selectivity. The response of the biosensor was tested for potential interferences by injecting aliquots of 100 μL of 0.1 mol L^{-1} PBS, and solutions of glucose, uric acid (UA) and ascorbic acid (AA). As illustrated in Fig. 5, after the stabilization of the current signal, an addition of dopamine caused a current decrease as expected. After an addition of ascorbic acid it was observed a positive step in the current signal, which can be easily distinguished as being an inverse signal to the DA. Other interferent solutions showed no signal.

Table 2 shows the comparison of the present work with other published sensors for DA determination using electrochemical methodologies. The performance of the ITO/PB/$[PEI/(MAO\text{-}B/(1POPG{:}4DPPG)]_7$ sensor is comparable with other electrochemical sensors presented in the literature. However, its stability increased when compared to sensors without enzymes protected by liposomes. This result can be associated to the environment. Native MAO is found bound to the membrane of mitochondria in most cells and liposome mimics this environment. It can be observed that the proposed sensor exhibits similar sensitivity to the literature, but improvements in the system should be done to reduce the limit of detection and to increase the linear range. Notwithstanding, we believe that a study of different numbers of enzyme bilayers and different molecular architectures can result in a better analytical performance, preserving the high selectivity of enzymatic biosensors. Here, it was demonstrated successfully, in the first time the LbL assembled MAO-B films for analytical determination of DA, showing the enhancement of the sensor response when the enzyme is immobilized in liposomes.

4. Conclusions

In this work we demonstrated that the α-helix structure of the MAO-B enzyme was preserved when encapsulated in liposomes in the proportion of 1POPG:4DPPG. The LbL deposition of the free enzyme MAO-B and incorporated in liposomes was monitored by SPR. The amperometric results indicated a fast response to DA detection with superior sensitivity (0.33 ± 0.02 (μA cm^{-2})/(mmol L^{-1}), lower detection limit (0.86 ± 0.16 mmol L^{-1}) and higher stability to the biosensor composed by ITO/PB covered with $[PEI/(MAO\text{-}B/(1POPG{:}4DPPG)]_7$ indicating that the liposome helps the preservation of enzymatic activity. The interfering test showed a negative response to the tested solutions,

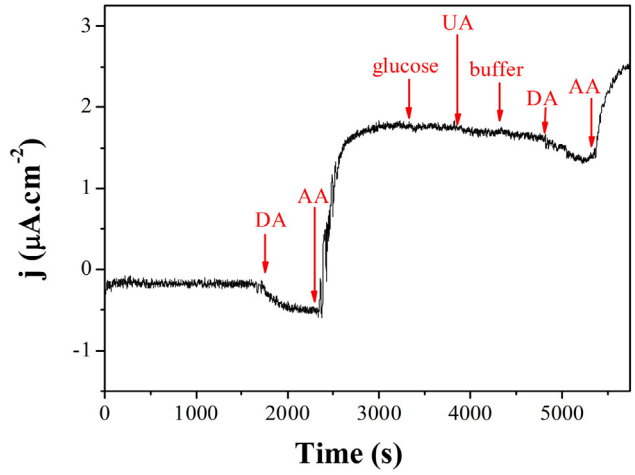

Fig. 5. Interferent effects of ITO/PB + $[PEI/(MAO\text{-}B/1POPG{:}4DPPG)]_7$ film in PBS (0.1 mol L^{-1} pH 7.4) at 0.0 V (vs SCE). Each addition corresponds to an injection of 100 μL of DA (dopamine), AA (ascorbic acid), glucose e UA (uric acid).

Table 2
Comparison with other published electrochemical sensors for DA detection.

Sensor	Method	Linear range (mol L^{-1})	LOD (mol L^{-1})	Response time (s)	Sensitivity (μA μmol L^{-1})	Ref.
Ferrocene encapsulated Pd-linked ormosil	Amperometry	N/A	5×10^{-5}	<20	1.25×10^{-4}	[52]
SWNT/NH-GME[a]	Amperometry	9.9×10^{-7}–3×10^{-4}	4.2×10^{-7}	N/A	60.4×10^{-6}	[53]
SiO_2/C/Cu(II) phtalocyanine[b]	Amperometry	10×10^{-6}–140×10^{-6}	6×10^{-7}	N/A	6.3×10^{-4}	[54]
Ppy/FCNMCPE[d]	LSV	1×10^{-4}–1.2×10^{-3}	3.86×10^{-5}	N/A	12.6×10^{-3}	[55]
	DPV	2×10^{-4}–9.5×10^{-4}	1.51×10^{-5}	N/A	38.6×10^{-3}	
ITO/PB/[PEI/(MAO-B/(1POPG:4DPPG)]$_7$	Amperometry	1.4×10^{-3}–1×10^{-2}	8.6×10^{-4}	45	3.3×10^{-4}	This work

[c] Prussian Blue and poly(3,4-ethylenedioxythiophene) electrochemically prepared onto Pt electrode.
[a] Single-walled carbon nanotube covalently attached to glass micropore electrode.
[b] Cooper phthalocyanine prepared on mesoporous carbon ceramic SiO_2/C.
[d] Carbon paste electrode modified with polypyrrole/ferrocyanide film.

except AA which demonstrated an inverse current signal to that of DA and was thus distinguishable from the signal provided by our analyte.

Acknowledgments

This work was supported by FAPESP (2014/15093-7 and 2014/17519-1), CAPES and CNPq (Brazil).

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