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Mushroom tyrosinase in polyelectrolyte multilayers as an optical biosensor for *o*-diphenols

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ABSTRACT

Determination of phenolic derivatives is very important in medical, food and environmental samples because of their relevant significance in health care and pollution monitoring. Tyrosinase-based biosensors are promising tools for this purpose because of several advantages with respect to currently used detection methods.

A key aspect in the development of a biosensor is the effective immobilization of the enzyme. In this work, ordered tyrosinase films on an optical transparent support were immobilized by a "layer-by-layer" (LbL) assembly, alternating the enzyme with the polycation polymer poly(dimethyldiallylammonium chloride). As confirmed by UV–vis spectroscopy, the LbL deposition allowed a high loading of enzyme. The immobilized tyrosinase functionality was proven and its kinetic parameters were spectrophotometrically determined. The prepared biosensor was used to optically detect the *o*-diphenolic compound L-3,4-dihydroxyphenyl-alanine (L-DOPA) and exhibited good repeatability and time stability. The sensing properties of the system were studied by means of both absorption and fluorescence spectroscopy. The bioassay based on the absorbance measurements gave a LOD of 23 μM and a linear response up to 350 μM. The bioassay based on the fluorescence measurements gave a LOD of 3 μM and a linear response in the range of tens of micromolar (the exact value depends on the number of mushroom tyrosinase layers). Biosensor sensitivity could be modulated varying the number of the immobilized enzyme layers.

1. Introduction

Phenolic compounds determination in medical and environmental matrices is of relevant interest because of their involvement in health care and pollution monitoring (Puig and Barceló, 1996). Phenolic derivatives may be natural or industrial products and they may have either beneficial or negative effects on human health and/or environment. In fact many studies have been aimed to determine the effects of these compounds on the health and to develop analytical methods for their detection and quantification (Meulenberg, 2009). Currently used methods (e.g. gas and liquid chromatography) are expensive (requiring costly equipment) and time-consuming (requiring the prior purification of the sample) (Chriswell et al., 1975; Wang et al., 2000; Meulenberg, 2009).

A need has arisen for a fast, sensitive and inexpensive detection method to monitor phenolic compounds and a substitution of analytical methods with a biosensor should be very useful. Tyrosinase-based biosensors have been proven to be promising tools for this purpose, because of their advantages such as selectivity, low cost, fast and cheap screening of samples. Tyrosinase (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) is a copper-enzyme which in presence of oxygen catalyzes two different reactions: the *o*-hydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols into reactive *o*-quinones (catecholase activity) (García-Borrón and Solano, 2002). Subsequently, the *o*-quinones undergo non-enzymatic reactions, producing intermediates which associate spontaneously in dark, red or brown pigments (Simon et al., 2009). In animals, plants and lower organisms tyrosinases are involved in the melanin pathway. Tyrosinase is responsible for the first step of melanin pathway, starting from L-tyrosine as substrate or L-3,4-dihydroxyphenyl-alanine (L-DOPA) leading to the formation of L-dopachrome (Simon et al., 2009) (Scheme SI-S1, in the Supporting Information). Since this reaction is associated to drastic spectral changes it could be used in simple optical bioassays. A common drawback of enzymatic bioassay is the consumption of expensive enzyme molecules. Accordingly, considerable effort was spent to immobilize functionally active tyrosinase thus allowing for the reuse of the enzyme.

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Many materials, such as polymers (Consnier and Innocent, 1993), alumina sol–gels (Liu et al., 2000), graphite–Teflon composite (Serra et al., 2002), MWNT–Nafion nanobiocomposites (Tsai and Chiu, 2007) magnetic nanoparticles–chitosan nanocomposites (Wang et al., 2008) have been employed to immobilize tyrosinase in biosensors. Most of these were used in amperometric biosensors where the enzyme was immobilized on the electrode surface and the current associated to the reduction of the enzymatically generated quinones was recorded. To the best of our knowledge, only two optical biosensors based on immobilized tyrosinase have been proposed, differing on the supporting membrane; viz nylon membrane (Russell and Burton, 1999) and chitosan (Abdullah et al., 2006). Note that in these latter two cases the optical detection was based on absorbance changes and requires the addition of an exogenous compound (3-methyl-2-benzothiazolinone hydrazone: MBTH).

Enzymes can also be immobilized in ordered protein films on a transparent solid support by means of the alternate deposition of polyanions and polycations: this leads to the formation of the so-called polyelectrolyte multilayers (PEM). Such films are readily prepared by "layer-by-layer" (LbL) assembly, a simple process based on the sequential adsorption, driven by electrostatic interactions, of cationic and anionic species on a charged support (Decher and Schlenoff, 2003; Schonhoff, 2003a,b). This technique has been used to prepare ordered protein films by alternating polyion layers with a number of different positively or negatively charged soluble proteins (Lvov et al., 1995). The obtained surfaces are transparent in UV and visible regions and they can be used in optical detection methods.

In the present paper, we show for the first time that mushroom tyrosinase (MT) can be incorporated in polyelectrolyte multilayers with a cationic polymer, poly(dimethyldiallylammonium chloride) (PDDA), and that its functionality can be preserved. The PDDA–MT multilayer has been deposited onto a quartz slide and the enzyme catecholase activity has been optically probed using L-DOPA as phenolic model substrate without addition of any exogenous reagent. Both absorbance and fluorescence changes have been successfully used to measure the rate of enzyme kinetics and the sensing properties of the system are investigated in view of its use in the design of a simple reagent-less optical biosensor for *o*-diphenolic compounds.

2. Experimental

2.1. Reagents

Lyophilized mushroom tyrosinase from *Agaricus bisporus* (MT) (EC.1.14.18.1; 4187 U/mg), PDDA and L-DOPA were purchased from Sigma–Aldrich. All other chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared with water obtained by a Milli-Q Gradient A-10 system (Millipore, 18.2 MΩ cm^{-1}, organic carbon content ≤4 μg/l). All enzyme and L-DOPA solutions, as well as enzyme electrostatic adsorptions and enzymatic reactions, were performed in a 20 mM Na–phosphate buffer, pH 7.0.

2.2. Apparatus

Absorption spectra were recorded by an Agilent 8453 diode array. Kinetic measurements at fixed wavelength were performed using a PerkinElmer Lambda-40 UV–vis spectrophotometer. For fluorescence measurements a Varian Cary Eclipse fluorimeter was used. For measurements, the quartz plate supporting the PDDA–MT multilayer was placed at 45° with respect to the measuring beam (in the spectrophotometer) and to both the emission and the excitation direction (in the fluorimeter) by inserting the plate along the diagonal of a 1 cm path length fluorescence cuvette, containing 2.5 ml of solution. All measurements were performed at (25 ± 1) °C.

2.3. Procedures

2.3.1. Preparation of PDDA–MT multilayers

The assembly by LbL adsorption of tyrosinase and PDDA was performed on an optical quartz support (1 cm × 5 cm slide, Hellma). Tyrosinase from *Agaricus Bisporus* has got an isoelectric point of 4.1 or 4.3, depending on the isolated isoform (Espín and Wichers, 1999). The pH of the protein solution has been set to 7.0, in this condition the enzyme is negatively charged and it electrostatically interacts with the positively charged PDDA.

Before initiating adsorption steps, the quartz slide was negatively charged by using an oxidative cleaning in a freshly prepared "piranha" solution (oleum H_2SO_4 and 30% H_2O_2, 3:1) for 15 min on ice (care should be taken in preparing and handling this solution, as the reaction is exothermic and the solution is highly corrosive) and subsequently left in water for 5 min. Finally, the quartz support was rinsed in acetone and dried with nitrogen. The adsorption steps were performed by incubating the slide at 4 °C within 1 cm × 1 cm cuvette containing 2.5 ml of solution. In particular, to adsorb the first polycation layer, the slide was immersed in a 2 mg/ml PDDA/water solution for 30 min. Subsequently, it was washed in water for 2 min, and a layer of negatively charged tyrosinase was adsorbed by dipping the slide into a 2 mg/ml MT/buffer solution for 30 min. After rinsing with water, the above described adsorption steps were repeated in order to obtain the required number of PDDA–MT layers. In this way, both sides of the slide are covered with the same number of PEM.

In the following, we will define the multilayer obtained by means of this procedure by the number of PDDA–MT alternate layers grown on one side of the quartz slide (i.e. by the number of immersions in the MT solution). To reduce the possibility of tyrosinase desorption, the last layer was always PDDA. After deposition the quartz-supported PDDA–MT multilayer was stored in buffer at 4 °C.

2.3.2. Assay of tyrosinase activity

DOPA oxidase (catecholase) activity was spectrophotometrically probed using L-DOPA as substrate and recording the increase in absorbance at 475 nm due to dopachrome formation (ε = 3700 M^{-1} cm^{-1}) (Mason, 1948; Solano and García-Borrón, 2006) over the first 2 min. The activity was also followed by fluorescence, using an excitation radiation of 285 nm and measuring the initial rate of L-DOPA emission decrease at 318 nm.

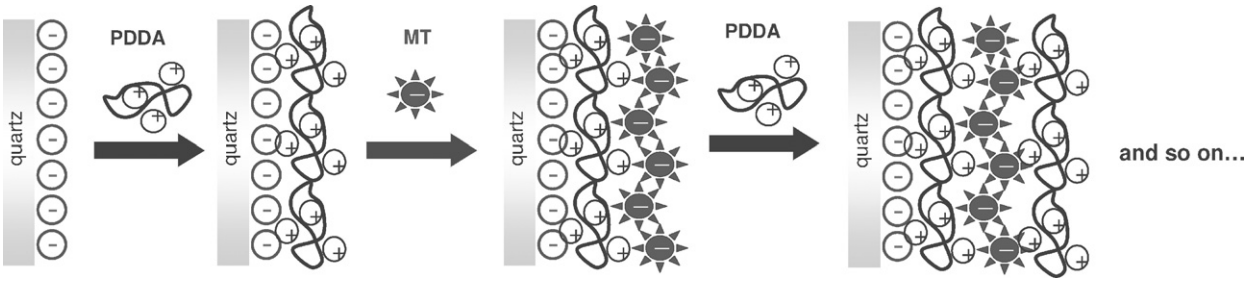
The reactions were started by inserting the multilayered quartz slide into the cuvette containing 2.5 ml of buffer solution with the suitable amount of substrate. After each measurement the multilayered quartz slide was washed several times in Na–phosphate buffer, pH 7.0.

3. Results and discussion

3.1. Preparation of PDDA–MT multilayers

The main driving forces for LbL assembly are electrostatic interactions. When a solid support (quartz slide) with negatively charged surface is immersed in a solution containing PDDA, a layer of polycation is adsorbed *via* electrostatic attraction. A number of cationic groups remain exposed to the solution, and thus, the surface charge is effectively reversed. After being rinsed in water, the support is immersed in a solution of tyrosinase resulting in the protein adsorption and in the re-establishment of a negative charge density. By repeating these steps, alternating multilayer assembly was obtained (see Scheme 1).

The efficiency of MT assembly by LbL technique has been estimated during the sequential stages of the adsorption process by recording the UV–vis spectrum in water from 200 to 500 nm. As



Scheme 1. Schematic of the LbL assembly of a PDDA–MT multilayer. For the sake of simplicity only deposition onto one side of the quartz slide is shown.

reported in Fig. 1, in our system, the optical density at all the wavelengths grows with the number of adsorption steps. To probe the growth of protein layers, in each spectrum we have subtracted the absorbance at 500 nm (where the MT absorbance is very small) from the absorbance at 258 nm (maximum in MT absorbance spectrum). As shown in the inset of Fig. 1, the enzyme absorbance grows exponentially with the number of adsorption cycles. This result is in agreement with data obtained by Mallardi et al. (2007), in fact an exponential growth of protein layers was observed when a different protein (a photosynthetic reaction center) was immobilized with PDDA in a PEM.

3.2. Functionality of the MT in PEMs

The biochemical properties of tyrosinase and its kinetic behaviour have been described in detail in literature (Simon et al., 2009). The catecholase activity of tyrosinase can be measured spectrophotometrically by means of a rapid and easy assay based on the measure of dopachrome formation (Mason, 1948; Solano and García-Borrón, 2006). Using this assay, the kinetic behaviour of the multilayered MT has been studied, determining the maximum reaction rate (V_{max}) and the Michaelis–Menten constant (K_m) of the immobilized enzyme. Fig. 2 shows the change of the absorption spectrum of a L-DOPA solution after the immersion in the cuvette of a quartz-supported PEM containing the enzyme. The spectra evidence the formation of an absorbance peak around 475 nm, which indicates the formation of dopachrome (Scheme SI-S1, in the Supporting Information). The initial rate of increase in absorbance at 475 nm is proportional to the initial rate of dopachrome formation and it can be used as measure of the extent of dopachrome formation (inset of Fig. 2).

Such a strategy of detection can be easily extended to others *o*-diphenols such as dopamine, catechol, caffeic acid and others because the formation of the oxidation products gives rise to a distinctive absorption band in the range 360–475 nm (see Selinheimo et al., 2009 for a list of the adsorption maxima of different *o*-diphenols).

The reuse of the same PDDA–MT multilayer is possible because rinsing it with buffer allows the removal of all the new formed dopachrome and of the unreacted L-DOPA. The typical hyperbolic growth and saturation upon increasing the substrate (L-DOPA) concentration is evident in the inset of Fig. 2. Accordingly, the data have been fitted by nonlinear regression to the Michaelis equation. The best fit values obtained are: $K_m = (0.76 \pm 0.07)$ mM and $V_{max} = (0.193 \pm 0.005)\ \Delta Abs\ min^{-1}$ (corresponding to $(52.2 \pm 1.4)\ \mu M\ min^{-1}$ of dopachrome formation). The K_m obtained value is in good agreement with the reported value for MT from *Agaricus Bisporus* in solution (Espín et al., 1997), indicating that the immobilized MT fully retained its affinity for the substrate. The high V_{max} value obtained should be due to the high enzyme amount embedded in the multilayer.

Our data demonstrate that the LbL assembly is a mild process that does not affect the functionality of the mushroom tyrosinase.

3.3. Sensing performance

The proposed assay is based on the relation between the (MT-catalysed) reaction rate and the substrate concentration. The maximum attainable rate (V_{max}) depends on the amount of enzyme molecules, which is a function of the number of assembled MT layers. Accordingly, we expect a dependence of the enzymatic activity on the number of assembled MT layers. To have insight on this point, the initial rate of dopachrome formation (enzymatic activity) has been determined during the quartz-supported PEM assembly. The results shown in Fig. 3 indicate that the enzymatic activity increases with the number of adsorbed MT layers. In particular,

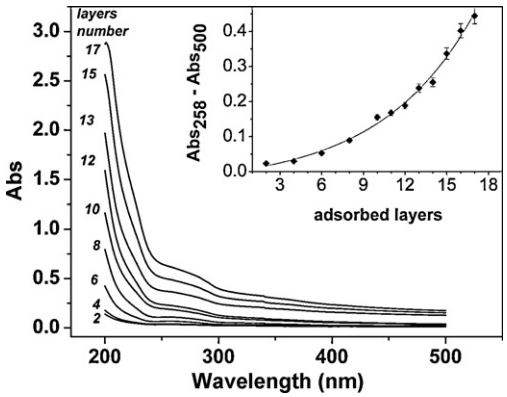


Fig. 1. UV–vis absorption spectra of PDDA–MT assembly by LbL technique, obtained following the sequential stages of the adsorption process. On the left of each spectrum the number of the layers is reported. Inset: difference between absorbances measured at 258 and 500 nm as a function of the number of MT adsorbed layers. Solid line represents the exponential fit.

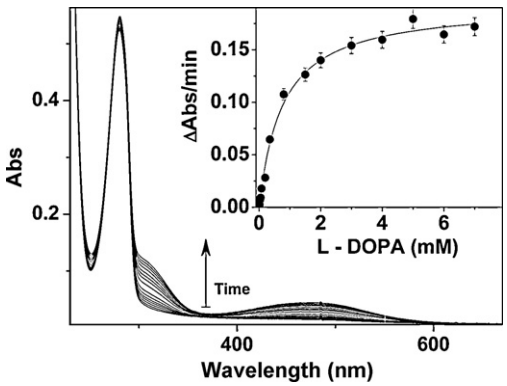


Fig. 2. Time-evolution of the absorption spectrum of a L-DOPA solution oxidized by a 10 MT layers PEM. The reaction was followed over a period of 60 s, after the immersion in the cuvette of the quartz-supported PEM. L-DOPA concentration was 200 μM. Inset: initial rates of dopachrome formation obtained with the 10 MT layers PEM. Solid line represents the nonlinear best fit to the Michaelis equation.

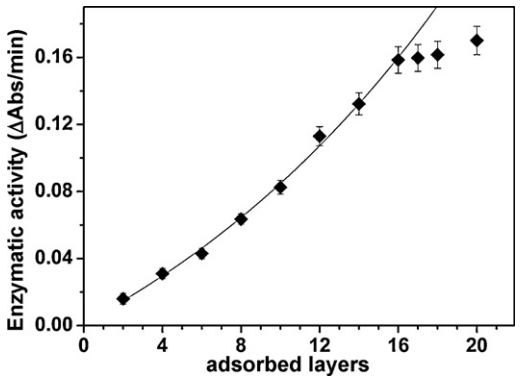


Fig. 3. Increase of the rate of dopachrome formation as a function of the number of adsorbed layers. L-DOPA concentration was 2 mM. Solid line represents the exponential fit.

the activity grows exponentially with the number of layers at least up to 16 layers; for a higher number of layers the growth becomes less steep. This result likely reflects the exponential increase in the protein optical density (and thus concentration) shown in the inset of Fig. 1. This finding suggests that the construction of sensors with different analytical performances could be realized through a different number of attached PDDA–MT layers. Since the measured enzymatic activity follows the Michaelis equation, the condition for linearity is attained when the analyte concentration is negligible in comparison with the K_m. Under this condition, the activity is proportional to the analyte concentration with a proportionality constant (sensitivity) equal to V_{max}/K_m. Thus the sensitivity can be conveniently modulated by controlling the enzyme loading (*via* the number of attached layers) and thus the V_{max}. This might be one of the most important advantages of the proposed method of immobilization.

With the aim to test the effectiveness of this system and in view of potential application as biosensor for *o*-diphenols, its analytical performances have been examined using L-DOPA, a typical *o*-diphenolic substrate. Generally speaking, *o*-diphenols are usually unsuited for end-point enzymatic assays based on tyrosinase because the resulting *o*-quinones are very unstable and undergo to a sequence of non-enzymatic reactions (polymerizations included) that preclude any isolation of an end product. To use the end-point approach one is forced to add MBTH or other nucleophilic compounds able to produce a stable stoichiometric adduct with the *o*-quinones (García-Molina et al., 2007). In the case of the MT immobilized on the quartz-supported PEM a further complication arises: the insoluble polymers (synthetic melanin) formed at later stage of the reaction adsorb strongly on the PEM that cannot be reused. In view of these drawbacks the detection of L-DOPA was accomplished by measuring the initial rate of dopachrome formation (or L-DOPA disappearance, see below) over the first 2 min of reaction. This is a time short enough to avoid the melanin formation and the PEM can be efficiently rinsed and reused.

The quartz-supported PEM has been used as the active component in spectrophotometric and fluorimetric determination of the reaction rates.

Fig. 4A shows a representative calibration plot of the initial rate spectrophotometrically determined. As shown, a linear response of the biosensor has been observed for substrate concentrations up to 350 μM. The data have been fitted to a straight line and the limit of detection (LOD) was calculated according to Miller and Miller (2000):

$$\mathrm{LOD} = \frac{(3 \times S_{y/x})}{a}$$

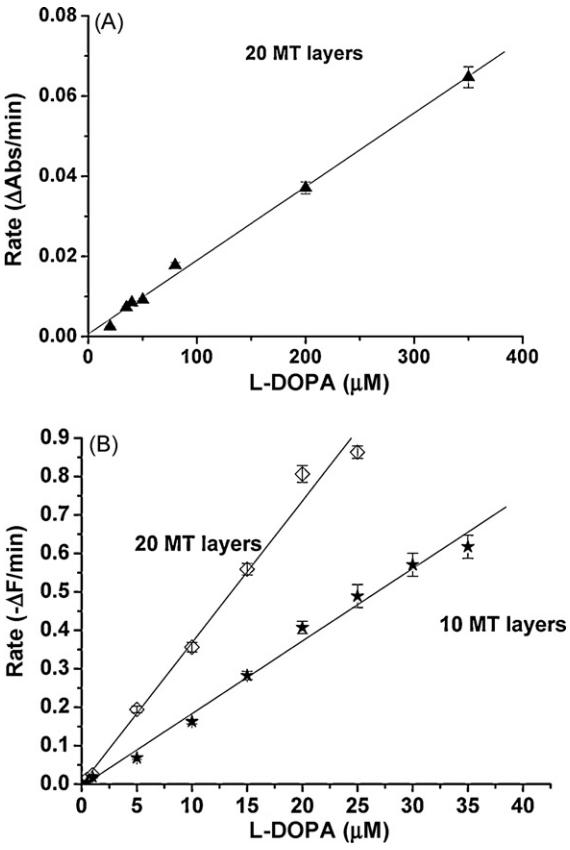


Fig. 4. Calibration plots of dopachrome formation vs. [L-DOPA]: (A) spectrophotometrically obtained using a 20 PDDA–MT multilayer; (B) fluorimetrically obtained using both a 20 and 10 PDDA–MT multilayers (the excitation wavelength was 285 nm and the emission wavelength was 318 nm).

where *a* is the sensitivity of the method (the slope of the calibration curve) and $S_{y/x}$ is the standard deviation of the fit. The sensitivity and the LOD obtained are 0.184 Abs min^{-1} mM^{-1} and 23 μM, respectively. The LOD value is very close to that reported from Solná and Skládal (2005) for a mushroom tyrosinase. These authors obtained for L-DOPA a LOD of 29 μM, using an amperometric flow-injection determination. However, our LOD is one order of magnitude higher than that (amperometrically obtained) reported from for a *Amorphophallus companulatus* tyrosinase (1 μM), using L-DOPA as substrate (Tembe et al., 2006).

It should be noted that for few micromolar L-DOPA concentrations the increase in absorbance of the produced dopachrome in the first few minutes of reaction is almost negligible and the initial rate could not be determined.

An improvement in the sensing performance has been obtained using fluorescence to evaluate the reaction rate. L-DOPA is a strongly fluorescent molecule (as many phenols) that can be excited in the UV and emits fluorescence centered around 318 nm (see Fig. SI-F1, in Supporting Information, for representative excitation and emission fluorescence spectra).

The MT-catalysed transformation of L-DOPA in dopachrome is associated to a drop in the L-DOPA fluorescence emission (and this is expected to hold for all the diphenolic substrates because the quinones have usually negligible fluorescence). Accordingly, the catecholase activity of MT was probed by monitoring the decrease of L-DOPA fluorescence emission at 318 nm (the excitation wavelength was fixed at 285 nm). The main advantage of fluorescence detection compared to absorption measurements is the greater achievable sensitivity, because the fluorescence signal has a very low background. In addition, as the assay is based on

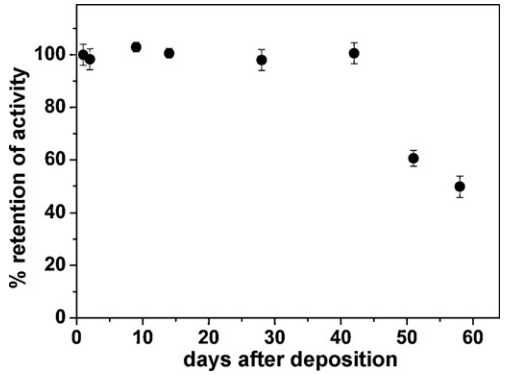


Fig. 5. Time stability of a 10 PDDA–MT multilayer. The value of 100% is referred to the catecholase activity measured as initial rate of dopachrome formation, with a freshly prepared multilayer. Its corresponding value of dopachrome produced is $(35.5 \pm 0.7)\,\mu M\,min^{-1}$, using a [L-DOPA] = 2 mM. Replicates: five for the point at time $t = 0$ and three for all the other points.

the measure of substrate disappearance, the highest fluorescence signal is observed in the first minutes of reaction, when the initial rate is determined, thus giving an accurate measure of the enzyme activity.

According to these considerations, the fluorimetric determination of the catecholase activity has shown to considerably improve the limit of detection for L-DOPA. Representative calibration plots are shown in Fig. 4B, where the performances of PEMs made of 10 or 20 MT layers are compared. The calculated LODs for the two PEMs are respectively 4 and 3 μM, one order of magnitude lower than the spectrophotometrically obtained values. For the 10 layer PEM a linear response of the biosensor has been observed for substrate concentrations up to 35 μM, while for the 20 layer PEM a linear response has been observed for substrate concentrations up to 25 μM. The sensitivity strongly depends on the amount of enzyme embedded in the PEM, the sensitivity for the 20 layer PEM being twofold higher than that found for the 10 layer PEM.

3.4. Stability and reproducibility

The PDDA–MT PEMs have shown to maintain their spectral features as well as activity over a period of at least one month. During this period each PDDA–MT PEM has been used for at least 40 assays furnishing consistent results. The long-term stability of the enzymatic activity of MT PEM has been checked measuring weekly the initial rate of dopachrome formation for constant initial L-DOPA concentration (2 mM) over a time interval of two months. When not in use, the PDDA–MT multilayer was immersed in buffer and kept at 4 °C. As shown in Fig. 5, the enzymatic activity remains almost unchanged (within 4%) during the first 6 weeks. Afterwards, a decrease of activity has been observed. According to our data the interaction of the enzyme with the support enhances the enzyme stability with respect to the enzyme in solution (Kertesz and Zito, 1965).

The repeatability of the same PDDA–MT multilayer has been examined by measuring the enzymatic activity for ten successive determinations, using L-DOPA at 2 mM concentration. A relative standard deviation of 2% was obtained, indicating a good repeatability of the measurements.

4. Conclusions

We developed a method to prepare an optical biosensor for detection of phenolic compounds by alternate LbL deposition of mushroom tyrosinase and PDDA on a quartz slide. The analytical performances of this biosensor have been examined using the L-DOPA as archetype substrate (analyte) but the biosensor is expected to work with others *o*-diphenolic compounds.

Immersion of the multilayered quartz slide into a diphenol solution catalyses its oxidation. Such a reaction can be easily probed without the use of exogenous reagents (e.g. MBTH) following absorbance or fluorescence changes. The same quartz-supported PEM can be used indifferently as the active element for absorbance and fluorescence assays. The quartz-supported PEM shows good time stability and can be rinsed retaining its catalytic activity thus allowing the reuse of the same multilayered support. The use of such a quartz-supported PEM in connection with commercial spectrophotometers or spectrofluorimeters provides a reagent-less biosensor for *o*-diphenols to laboratories lacking specific electrochemical skills. The sensor performances can be tuned to the desired level by controlling the number of attached enzyme layers and also by using two different optical techniques on the same quartz-supported PEM.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.01.033.

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