ARTICLE

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Peroxidase Immobilized on Phospholipid Bilayers Supported on Au (111) by DTT Self-Assembled Monolayers: Application to Dopamine Determination

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ABSTRACT: In this work, horseradish peroxidase (HRP) was immobilized on dimyristoylphosphatidylcholine (DMPC) bilayers supported on Au (111) by dithiotreitol (DTT) self-assembled monolayers and used as a nanostructured electrochemical biosensor to dopamine determination. The morphology of the phospholipid bilayers and the immobilization of HRP to these layers were characterized by atomic force microscopy (AFM). Square-wave voltammetry (SWV) experiments were done to investigate the performance of the HRP-modified electrode. The AFM images indicate that the enzyme is adsorbed at the external layer of the lipid bilayer and, although the electrical charges on the surface were not measured, the enzyme and phospholipids surface interaction occurs probably by electrostatic forces due to the pH used in the experiments. Interestingly, the present system can be used as one-shot sensor for the rapid detection of dopamine. The analytical performance of this system was linear for dopamine concentrations from 3.3×10^{-5} to 1.3×10^{-3} mol L^{-1} ($r = 0.9997$) with a detection limit of 2.0×10^{-6} mol L^{-1}. Our results indicate that the use of HRP-DMPC bilayer system may be useful not only in developing new nanostructured materials for technological purposes, but could be very useful in fundamental studies to investigate the interactions between different micro-and macromolecules, even with soluble proteins, and lipid membranes.

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KEYWORDS: lipid bilayers; peroxidase; dopamine; self-assembled monolayers; AFM; electrochemistry

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Introduction

Lipid bilayers supported on solid materials have been extensively used as model systems for cell membranes, as started by Tamm and McConnell (1985). In parallel, several attempts have been made to use enzymes immobilized on lipid bilayers supported on solid materials for the fabrication of biosensors (Castellana and Cremer, 2006; Janshoff and Steinem, 2006; Xu et al., 2006). Compared with other materials, lipid bilayers may provide a natural environment for immobilizing enzymes since phospholipids are major components of the cellular membranes. Furthermore, the presence of the lipid bilayer greatly reduces background noise (interferences) and effectively excludes macromolecules that could block the electron transfer of proteins and hydrophilic electroactive compounds from reaching the detection surface (Xu et al., 2006).

To make electrochemical measurements with lipid bilayers immobilized on conductive solid supports, it is very common to use noble metal surfaces as working electrode such as gold (Au) supports. However, gold surfaces should be functionalized in order to promote vesicle fusion when preparing supported lipid membranes; since cleaning procedures, roughness and crystallographic defects may confer hydrophobic nature in such material (Knoll et al., 2008). The Au (111) surface is the most commonly used for studies involving the modification of surfaces by an organic monolayer (Love et al., 2005; MacDairmid et al., 2003). The oriented Au (111) surfaces are obtained by submitting as deposited Au films to flame annealing process. For metals such as Au, which have a face-centered cubic packing, the flame annealing process causes a rearrangement of atoms in the hexagonal surface, making it smooth and enlarging the grain size.

A promising approach is to use SAMs of thiol molecules on gold due to the easy preparation, low cost and well-defined order (Chen and Li, 2006; Love et al., 2005). Thiols monolayers are spontaneously adsorbed on the surface of a conductor support based on the strong interaction between the metal and sulfur molecule. These monolayers are usually formed by immersing the conductive solid support pre-treated in solutions containing millimolar concentrations of thiols. The formation of monolayers of self-assembled organosulfur molecules has attracted much attention due to their technological applications (Mandler and Turyan, 1996; Phong et al., 2005; Sawaguchi et al., 2001).

Our research group has been working with the dithiotreitol (DTT) as a sulfur bridge to separate lipid bilayers from Au surface. The DTT is a short α,ω-alkanedithiol with two hydroxyl groups that may provide an OH-rich surface, facilitating the lipid bilayers formation. Creczynski-Pasa et al. (2009) observed that the DTT molecule can self-organize on the Au (111) surface in a "lying-down" configuration, irrespective of the concentration and temperature. The Au surface modified with DTT monolayer allows the formation of fluid dimiristoilfosfotidilcolina (DMPC) bilayer domains by vesicle fusion as revealed by in situ AFM images. The Au (111)—DTT—DMPC system was used to test the electrochemical influence of methylene blue (MB) and flavin adenine dinucleotide (FAD) on the charge transport through the lipid membrane (Creczynski-Pasa et al., 2009).

The peroxidase immobilization has been an interesting option to model systems for enzymes immobilized on supported lipid bilayers. The interactions between the enzyme and lipid bilayers can be a combination of hydrophobic, dipole, and electrostatic forces (Liu et al., 2006; Schmidt et al., 2008). Although peroxidase is not a membrane protein, this enzyme can form organized proteo-lipid nanostructures and the lipid bilayers may provide the microenvironment for the study of redox proteins, as well as for application in biosensors and catalysis. Horseradish peroxidase (HRP) is a heme containing glycoprotein (FW ca. 44 000) and it is the most commonly used enzyme for practical analytical applications, mainly because it retains its activity over a broad range of pH and temperature (Veitch, 2004). The peroxidase catalytic cycle occurs through a multistep reaction that involves, first, the reaction of the enzyme active site with hydrogen peroxide to form an unstable intermediate. In this latter state of the protein, the formed compound oxidizes an organic substrate (electron donor), generating a substrate radical and the compound II, where the organic cation radical is reduced by a second substrate molecule, regenerating the native form of the enzyme. Phenolic compounds can act as electron donor in the peroxidase reaction with hydrogen peroxide, which is the principle of using peroxidase-modified electrodes for the detection of phenolic species, among others (Castilho et al., 2005; Regalado et al., 2004).

Dopamine is an important naturally occurring neurotransmitter in the mammalian central nervous system. A deficiency in this catecholamine level can cause some serious diseases such as Parkinson, epilepsy and senile dementia in humans (Smith and Devlin, 1992). There is considerable interest in the quantitative determination of this neurotransmitter in human physiological fluids in biochemical and clinical diagnoses, as well as on dopamine solutions, in order to titrate the concentration after preparation and to testify the quality control of pharmaceutical products. To develop a cheap sensor to detect dopamine concentrations for diagnosis of diseases related to low production of the catecholamine is a challenge; a clinical, analytical screening method to diagnose Parkinson disease for example does not exist. One of the primary challenges is that the concentration of dopamine in the extracellular fluid of the caudate nucleus is extremely low (0.01–1 μ mol L^{-1}) for a healthy individual and in the nanomolar range for patients with Parkinson's disease (Robinson et al., 2008).

The conventional dopamine detection methods existing such as Enzyme-Linked Immunosorbent Assay (ELISA) kits are based on antibody/antigen reactions. ELISA kits for dopamine detection available commercially present high sensitivity and detection limit even lower then 5 ng mL^{-1}. However, kits based on antibodies also bring some limitations, including possible cross-reactions according to the manufacture's guides, besides the price and the laborious procedures. By another side, techniques such as positive-ion electrospray tandem mass spectrometry, which is a combination of HPLC with ESI–MS/MS developed to detect catecolamines, including dopamine when they are overproduced in biological samples, shows a quantification limit around 2.5 μg mL^{-1} (Kushnir et al., 2002).

Selective and sensitive determination of dopamine has been a long-standing goal, and electrochemical techniques have been proved to be one of the most advantageous ways in the determination of dopamine (Caruso et al., 1999; Fritzen-Garcia et al., 2009a; Lupetti et al., 2005; Ma and Sun, 2007).

In the present work, we used electrochemical techniques and atomic force microscopy to evaluate the interaction of HRP with phospholipid bilayer supported on Au (111) by

DTT self-assembled monolayers. The efficiency of HRP immobilization was investigated and this system was applied to determine the concentration of dopamine on prepared solution.

In the present work, we used electrochemical techniques and atomic force microscopy to evaluate the interaction of HRP with phospholipid bilayer supported on Au (111) by DTT self-assembled monolayers. The efficiency of HRP immobilization was investigated and this system was applied to determine the concentration of dopamine on prepared solution.

Materials and Methods

Chemicals

Dithiotreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) and horseradish peroxidase (HRP) were purchased from Sigma–Aldrich Co. (St. Louis, MO); dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama). Reference solutions of dopamine (Sigma–Aldrich Co.) were prepared by appropriate dilution of a 0.05 mol L^{-1} dopamine stock solution in 0.1 mol L^{-1} phosphate buffer (pH 6.5) and the hydrogen peroxide used was purchased from Merck Co. (Whitehouse Station, NJ). All other supplies used were of the best analytical grade commercially available and prepared using water from a Milli-Q system (Merck Millipore Headquarters, Billerica, MA) with resistivity not less than 18 MΩ cm.

Au Support Preparation

The Au support used to prepare the thiol self-assembled monolayers consisted of 250 nm of thick gold films prepared by physical vapor deposition on a 4 nm chromium layer on glass (Gold Arrandee®, Werther, Germany). A 3 min flame annealing process was performed by exposing the Au support to a butane flame until it reached the red color, which corresponds to approximately 650°C. This process generates a support consisting of atomically flat Au (111) terraces, separated by monatomic steps.

Self-Assembly and Characterization of DTT SAMs

SAMs of DTT were formed by immersion of Au (111) support in 50 μmol L^{-1} DTT ethanolic solution at 60°C within 30 min incubation time, as illustrated in Figure 1a, following the recipe in Creczynski-Pasa et al. (2009).

The DTT self-assembled monolayers were than analyzed by the acquisition of reductive desorption curves, following the procedure described elsewhere (Vela et al., 2000). Briefly, a potential sweep was applied to the DTT-covered Au support immersed in a three-electrode electrochemical cell containing 0.1 mol L^{-1} NaOH at 0.05 V s^{-1} from −0.4 to −1.4 V. The solution was freshly prepared and deaerated with purified nitrogen.

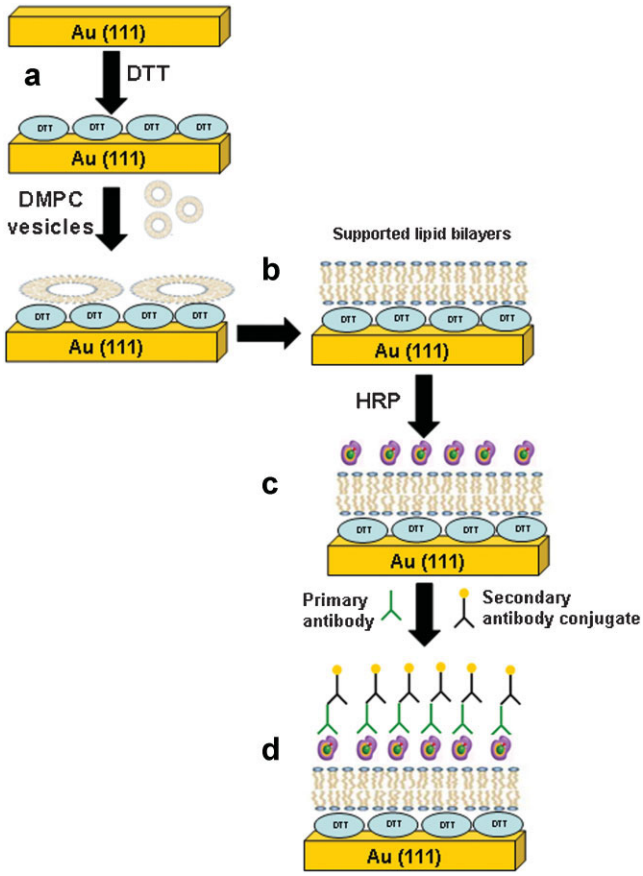

Figure 1. A schematic representation of the formation of DTT SAMs on gold (Au) support (**a**); with subsequent fusion of DMPC vesicles forming lipid bilayers (**b**); HRP immobilization on lipid bilayers supported on DTT/Au (**c**) and immunolabeling HRP immobilized on lipid bilayers (**d**).

Self-Assembly of DMPC Bilayers on DTT-Covered Au Support

DMPC vesicles were obtained by evaporating, under a stream of nitrogen, the solvent of a phospholipidic solution (20 mg mL^{-1}) prepared in chloroform. To eliminate traces of the solvent, the samples were dried under vacuum for 1 h. Subsequently, multilamellar liposomes were obtained by the addition of a buffer containing 0.01 mol L^{-1} HEPES + 0.9% NaCl (pH 7.4) and vortexing. The multilamellar suspension was then extruded through a polycarbonate filter with 400 nm pore (Nuclepore) to form unilamellar vesicles (Creczynski-Pasa et al., 2000; Olson et al., 1979). The liposomes obtained were diluted in buffer to a final concentration of 10 mg mL^{-1}.

DMPC bilayers were prepared by the vesicle fusion method in Au supports containing DTT monolayers, see Figure 1b, following the procedure indicated by Creczynski-Pasa et al. (2009). The supports were immersed in 10 mg mL^{-1} DMPC unilamellar vesicle suspension for 90 min at 30°C (above DMPC phase transition temperature $T_m = 23.9$°C). To remove the unbounded lipids, the

supports were rinsed with 0.01 mol L^{-1} HEPES + 0.9% NaCl (pH 7.4) and immediately used for electrochemical and AFM analysis.

HRP Immobilization on Lipid Bilayers Supported on DTT/Au

Different concentrations of HRP (0.05–2 mg mL^{-1}) were mixed with the DMPC vesicles suspension (10 mg mL^{-1}) and, about 20 μL of this mixture were added to DTT-covered Au supports. After 90 min of incubation, the functionalized Au supports were rinsed with HEPES 0.01 mol L^{-1} NaCl + 0.9% (pH 7.4) to remove the excess of liposomes and HRP not attached. After this step, the enzyme–lipid bilayer system, Figure 1c, was used for dopamine determination by electrochemical techniques and AFM measurements.

Immunolabeling HRP Immobilized on Lipid Bilayers

In order to prove the efficiency of HRP immobilization on lipid bilayers, the AFM was used to observe if the Au nanoparticles were attached to the receptors (see Fig. 1d) using a modified antibody protocol described by Palocci et al. (2007): DTT/Au supports containing lipid bilayers with HRP were preincubated for 5 min with 1% w/v bovine serum albumin (BSA; Sigma–Aldrich Co.) in phosphate buffer (0.1 M, pH 6.5) and then incubated for 30 min at room temperature with the mouse polyclonal antibody to HRP (ABCAM, Inc., Cambridge, MA) at a concentration of 40 μg/mL. After washing on phosphate buffer containing 1% w/v BSA, samples were labeled with anti-mouse IgG 10-nm gold nanoparticle conjugate diluted 1:10 (Sigma–Aldrich Co.) for 30 min at room temperature. Finally, samples were washed with phosphate buffer and observed by AFM.

Electrochemical Measurements

Cyclic and square wave voltammetry experiments were performed in a potentiostat/galvanostat Autolab PGSTAT30 (Eco Chemie, Utrecht, Netherlands) using a conventional three-electrode system with an Ag/AgCl (saturated 3 M KCl) as reference electrode, platinum wire as auxiliary electrode and DTT-SAM-covered Au support functionalized with lipid bilayers containing or not HRP as working electrode. The measurements were made in a 0.1 mol L^{-1} phosphate buffer (pH 6.5) containing 2.0×10^{-3} mol L^{-1} hydrogen peroxide at 25°C, as described in a previous work (Fritzen-Garcia et al., 2009a). The cyclic voltammograms were recorded by cycling the potential between −0.2 and +0.6 V at a scan rate of 100 mV s^{-1}. The square-wave voltammograms were obtained in the potential interval from −0.8 to +0.8 V at a frequency of 30 Hz and pulse amplitude of 50 mV after successive additions of the dopamine stock solutions. The dopamine standard curve was constructed by plotting the magnitude of the resultant current peak against the corresponding dopamine concentration.

AFM Measurements

The AFM images of HRP and DMPC bilayers systems were performed in situ at room temperature using a Molecular Imaging PicoSPM atomic force microscope (AFM; Tempe, AZ), with a 6 μm tube scanner. The images were obtained in contact mode, using oxide sharpened silicon nitride probes (Veeco NanoProbes, Santa Clara, CA) with cantilevers of 196 μm length, spring constant of 0.12 N/m, nominal tip radius of curvature of 10 nm, and applied forces were of the order of 3–10 nN. The scan line speed was optimized between 1 and 3 Hz with a pixel number of 512 × 512. Height and cross-sectional size measurements were carried out using the software WSxM version 4.0 from Nanotec Electronica, Spain (Horcas et al., 2007).

Results and Discussion

DTT molecules could provide a hydrophilic environment for anchoring different types of biomolecules. DTT-SAMs prepared in diluted solutions at 60°C, conditions that assure the lying down configuration of the molecules (Creczynski-Pasa et al., 2009) and can be used to form ordered phospholipidic bilayer domains on Au (111) surfaces. Reductive electrochemical curves displayed in Figure 2 were obtained to confirm the presence of DTT covalently attached to the Au (111) support. The cathodic polarization curve (a) shown in Figure 2 corresponds to a clean (thiol SAM-free) preferentially oriented Au (111) support recorded from −0.40 to −1.40 V. It is possible to observe in a very negative potential a cathodic increase of the current

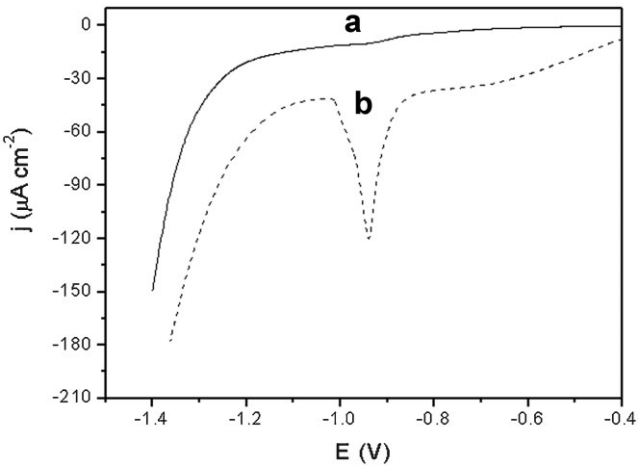

Figure 2. *j* versus *E* profiles for the reductive desorption of DTT SAMs. (a) bare Au (111) and (b) Au (111) incubated 30 min in DTT 50 μmol L^{-1} at 60°C. Measurements were performed in NaOH 0.1 mol L^{-1} at a sweep rate of 0.05 V s^{-1}.

density related to hydrogen evolution reaction (HER). The curve (b) in the figure shows the cathodic polarization behavior of DTT self-organized monolayer on Au (111), which corresponds to the reductive desorption of DTT according to the reaction:

$$\text{Au(111)-S-R} + 1e \rightarrow \text{R-S-} + \text{Au(111)} \qquad (1)$$

The cathodic peak potential (Epc) for DTT electrodesorption from Au (111) is observed at about −0.93 V. A typical potential for desorption of alkanethiols found in the literature is around −1.0 V (vs. saturated calomel electrode—SCE), but this value may vary depending on the chemical structure of the thiol and on pH (Kawaguchi et al., 2000). The potential value observed in Figure 2 is the same found by Creczynski-Pasa et al. (2009), which was expected since the same experimental condition was used. Furthermore, integration of the current involved in the electrodesorption peak gives the charge density (q) corresponding to the amount of chemisorbed species. By taking into consideration the reaction (Equation 1), where each electron is equivalent to one chemisorbed sulfur atom, and the actual area of Au support, it was possible to obtain a q of 72.6 $\mu C\,cm^{-2}$, consisting of DTT molecules chemisorbed in lying down configuration as previously measured by Creczynski-Pasa et al. (2009).

After DTT-SAM-covered Au samples were characterized by electrodesorption curves, the system was used to immobilize DMPC bilayers with or without peroxidase. To study the morphology of DMPC- and HRP-DMPC systems, AFM images were obtained by contact mode in-situ. Contact mode imaging is less suitable for soft or weakly attached materials, since the tip can often scrape or drag the sample surface during scanning, a disadvantage that can be overcome by applying intermittent methods. However, studies have also demonstrated that by adjusting the operative force it is possible to use contact mode to obtain AFM images of soft materials such as lipid membranes and colloidal systems (Fritzen-Garcia et al., 2009b; Munford et al., 2005).

Moreover, for biological samples as supported lipid membranes it is important to measure the surface topography inside liquid cells. The in-situ measurements have the additional advantage that permits the study of morphological modifications due to biological processes in real time and under physiological conditions. AFM contact mode is the convenient way for measuring samples immersed in a liquid. This operation mode avoids the necessity of having cantilever oscillating in a resonant frequency damped by the fluid.

Figure 3a shows the contact mode AFM image of Au Arrandee® after flame annealing process (Au (111)) and Figure 3b the contact mode AFM image of DMPC bilayers deposited on DTT-SAM-covered Au (111). It is possible to observe on the bright areas the formation of lipid bilayers and on the dark ones the not-covered surface. In the transition of covered and not-covered regions it was possible to measure the height of approximately 6 ± 1 nm, corresponding to a lipid bilayer. This value is slightly higher than the expected value for a DMPC bilayer, which is about 5 nm (Munford et al., 2005; Tang et al., 2002). This fact is probably due to the hydration of the DTT-DMPC interface.

AFM images were also obtained for HRP-DMPC system to investigate enzyme-lipid interactions. An antibody against HRP and an anti-antibody (IgG-10 nm gold nanoparticle) were used to pinpoint the enzyme on the phospholipid surface. Figure 4a shows a topographic image of HRP in the DMPC bilayer before the introduction of antibodies, while Figure 4b shows the image obtained after antibody reaction. Figure 4c displays a magnification of a specific area of Figure 4b and d line-scan profiles indicated in Figure 4a and c.

Before the antibody protocol was applied in the samples, numerous protrusions were observed corresponding to HRP molecules on the DMPC bilayers. The distribution of the enzyme on the bilayer was not uniform and the protrusions were formed probably due to the aggregation of HRP

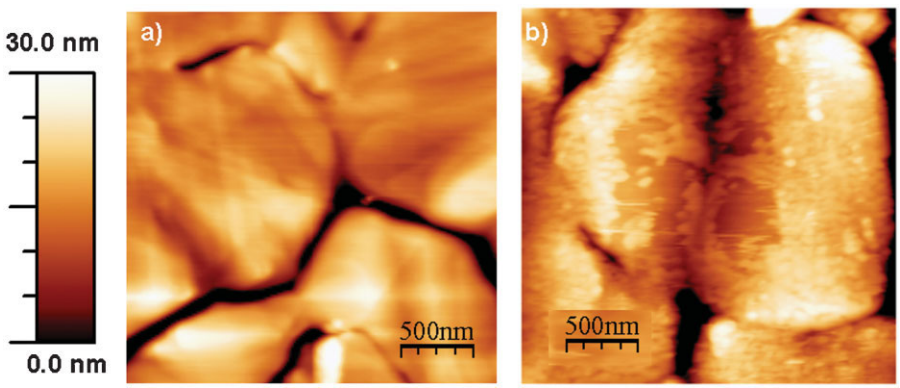

Figure 3. Contact mode AFM image (2.5 μm × 2.5 μm) obtained in-situ at 25°C temperature of Au (111) (**a**) and DMPC bilayer formed on DTT-SAM-covered Au (111) (**b**).

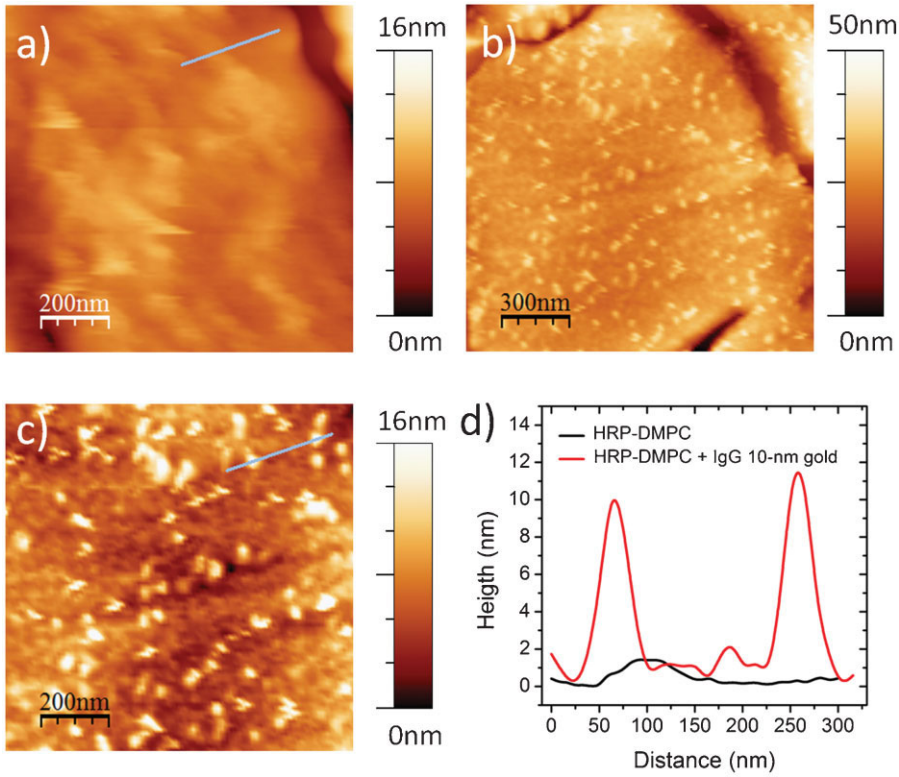

Figure 4. Contact mode AFM image of HRP-DMPC bilayer system before (**a**) and after labeling with antibody IgG 10-nm gold conjugate (**b**); magnification of a specific area of (b) (**c**) and line-scan profiles indicated in (a) and (c) (**d**).

molecules. The results indicate that the interaction between HRP molecules and lipid bilayer is relatively strong, since it was not possible anymore to observe the original surface with the lipid bilayers on Au, shown in Figure 3b, even after rinsing the samples with buffer solution. Similar structures were observed in the study of Tang et al. (2002), in which the conformational change of HRP on the DMPC bilayer directly supported on mica was investigated. These authors suggest that HRP is adsorbed spontaneously and is randomly distributed on DMPC bilayers surface. The enzyme adsorption changed drastically the domains and defects in the lipid layer, probably due to electrostatic interactions between enzyme and phospholipidic films.

Zhdanova and Kasemo (2009) studied the dependence of the rate constants of protein adsorption and desorption on the fraction of charged lipids. These authors discussed that protein-lipid electrostatic interaction may be energetically favorable if a lipid bilayer contains a significant amount of lipids with oppositely charged head groups.

Other study shows that HRP can retain its native state in dipalmitoylphosphatidic acid (DPPA), an anionic-type of lipid (Liu et al., 2006). The authors suggest that the HRP was entrapped in DPPA films and the results obtained indicate that DPPA membrane provides a mimic environment for the functioning of proteins, in which the native conformations of the proteins are retained and the electron-transfer rates are greatly enhanced compared with those involving proteins at bare electrode.

Although, we did not measure the electric charges on the surface, there is a possibility of such a surface to be negatively charged by the presence of the phosphate groups of the phospholipids. It is known that phosphatidylcholine heads of the phospholipids are zwitterionic and thus globally uncharged at neutral pH, but exhibiting a negative zeta (ζ) potential as the pH decreases (Garcia-Manyes et al., 2006). Concerning the HRP, its isoelectric point is 7.5 (Schmidt et al., 2008), and therefore the molecules of this enzyme were positively charged at the pH 6.5, the pH used in the experiments. Therefore, the HRP is adsorbed at the external layer of the DMPC bilayer probably mainly by electrostatic interactions.

In the Figure 4b, the presence of white spots is clearly evident. The height of the features is $\sim$10 nm above the lipid bilayer (Fig. 4d) and that is the diameter of the nanoparticle conjugated to the anti-IgG (anti-antibody), confirming the presence of HRP on lipid bilayer. The widths of the peaks in the line scan profile appear considerably larger than the nominal diameter of gold sphere due to the convolution between the shape of the AFM tip and the nanoparticle.

Since we have concluded with AFM that the enzyme was immobilized on the lipid bilayer, the electrochemical properties of the system were investigated by voltammetric

techniques and used for dopamine determination. Despite not having any work in the literature that uses such system for dopamine determination, our study aimed to verify this potential application in dopamine prepared solution.

The analytical performance of HRP-DMPC bilayer system was assessed by the voltammetric response obtained as a function of HRP concentration in the immobilization process. That is, it was investigated the effect of HRP concentration on the resultant current from the redox reaction of dopamine, in the electrode surface in the presence of hydrogen peroxide. There was no significant increase in the response with the HRP concentration greater than 0.5 mg mL^{-1} (results not shown) and therefore, this concentration was selected for further experiments.

The electrochemical properties of bare Au (111), DMPC bilayer and HRP supported on DTT-SAM-covered Au (111) and HRP-DMPC bilayers system were investigated by cyclic voltammetry in the presence of hydrogen peroxide and dopamine (Supplementary Material). The pair of peaks observed in the cyclic voltammograms corresponds to the redox process of dopamine in buffer solution at pH 6.5. The *o*-dopaquinone produced is electrochemically reduced back to dopamine at the electrode surface, generating currents that are directly proportional to dopamine concentrations. These results gave solid evidence that the lipid bilayer provided better conditions for peroxidase to catalyze the oxidation of dopamine into *o*-dopaquinone in the presence of hydrogen peroxide.

Afterwards the optimization of the assays conditions and the respective controls, square wave voltammetry, a more sensitive technique, was used to obtain the voltammograms of HRP immobilized on DMPC bilayers after successive additions of dopamine. The values of frequency and amplitude used in these experiments were 30 Hz and 50 mV, respectively. As described in the literature, using such protein for dopamine determination the response increases as the potential is changed to more positive values and reaches a maximum current intensity at 50 mV (vs. Ag/AgCl). On negative potentials an inactive form of HRP can be formed by the reduction of iron present in the active site of the enzyme from the state (III) to (II) (Santos et al., 2007). Above 50 mV the electric current may decreases considerably, since it may be more difficult for the reduction of quinone species. The influence of pH on the voltammetric response of the biosensor for dopamine determination was examined in the range between 6.0 and 8.0. It was possible to observe an increase of the electric current corresponding to dopamine reduction while the pH increases until a maximum value of 6.5. After that, the electric current decreases again (results not shown). Therefore, the pH 6.5 was used for subsequent studies and is consistent with pH value found in the previous work using a biosensor based on peroxidase immobilized on polymeric nanoparticles (Fritzen-Garcia et al., 2009a).

Figure 5a shows the square-wave voltammograms of the resultant current from the redox reaction of dopamine on the surface of the electrode containing HRP immobilized on DMPC bilayer supported on Au (111) by DTT SAMs. The sensor showed a linear response to different concentrations of dopamine (Fig. 5b) in the range of 3.3×10^{-5} to 1.3×10^{-3} mol L^{-1} ($r = 0.9997$) with a detection limit of 2.0×10^{-6} mol L^{-1}. The regression equation obtained was $\Delta i = 15.7 + 7.15 \times 10^{5}$ [dopamine], where Δi is the electric current measured in μA and [dopamine] is the concentration of dopamine in mol L^{-1}.

These results indicate that HRP immobilized in the DMPC bilayer supported on Au (111) by DTT SAMs can catalyze efficiently the redox reaction of dopamine. The concentration range found for dopamine in this study was similar to the result obtained in a previous work using a modified carbon paste electrode based on peroxidase obtained from pine kernel immobilized on polymeric nanoparticles (Fritzen-Garcia et al., 2009a). By comparing the electrochemical response of both electrodes, for the same dopamine concentration of 1.3×10^{-3} mol L^{-1}, we have observed: (i) the detection limit is almost one order of

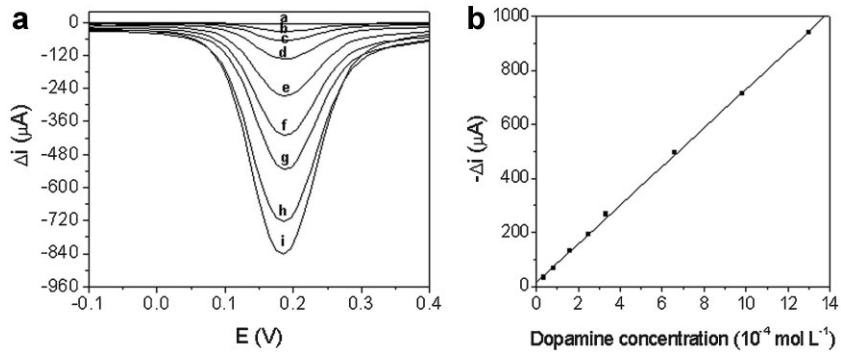

Figure 5. Square-wave voltammograms obtained using the electrode containing HRP (0.5 mg mL^{-1}) immobilized on DMPC bilayers (10 mg mL^{-1}) supported on Au (111) by DTT SAMs (**a**) and the analytical curve of the proposed electrode (**b**). Conditions: 0.1 mol L^{-1} phosphate buffer solution (pH 6.5) containing 2.0×10^{-3} mol L^{-1} hydrogen peroxide and dopamine solutions at the following concentrations: (b) 3.3×10^{-5} mol L^{-1}; (c) 8.4×10^{-5} mol L^{-1}; (d) 1.6×10^{-4} mol L^{-1}; (e) 2.5×10^{-4} mol L^{-1}; (f) 3.3×10^{-4} mol L^{-1}; (g) 6.6×10^{-4} mol L^{-1}; (h) 9.8×10^{-3} mol L^{-1}; (i) 1.3×10^{-3} mol L^{-1} at pulse height 50 mV, frequency 30 Hz and increment 2.0 mV.

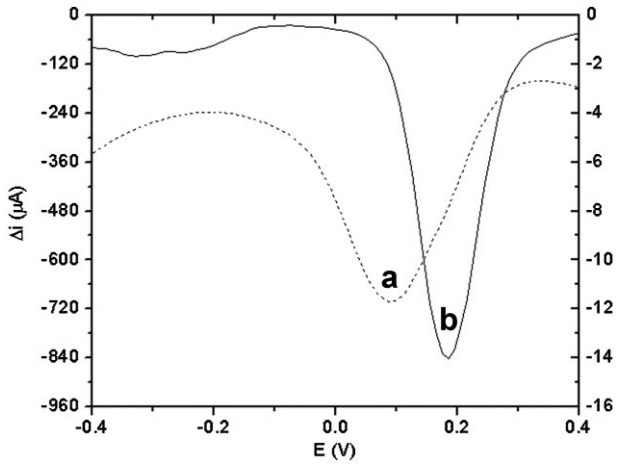

Figure 6. Comparison between the response of (**a**) a modified carbon paste electrode based on peroxidase immobilized on polymeric nanoparticles and (**b**) an electrode containing HRP immobilized in DMPC bilayer supported on Au (111) by DTT SAMs. The dopamine concentration was 1.3×10^{-3} mol L^{-1}. The arrows indicate the corresponding current scale for each curve.

magnitude lower for HRP on lipids (2.0×10^{-6} mol L^{-1}) than on polymeric nanoparticles (9.0×10^{-6} mol L^{-1}), probably due to the measured background that is much lower in the Au electrode and (ii) the voltammograms present significant differences concerning the width, potential position and intensity of the signal, as discussed below.

As can be seen in Figure 6, the modified gold electrode presents a much larger current than the one found with the modified carbon paste electrode, that is, about two orders of magnitude larger. This remarkable enhancement could be probably caused by the fact that the redox reaction of dopamine is facilitated by the planar Au (111) electrode, since in this case the surface area is greater than the modified carbon paste electrode used in the previous work (Fritzen-Garcia et al., 2009a). The voltammogram shown in curve (a) is two times wider than the one shown in curve (b), as measured considering the full width at half maximum (FWHM) of 200 and 100 mV, respectively. Note that the current peak recorded for carbon electrode is broader than that recorded for Au (111) probably due to the presence of different enzyme conformations. In addition, the peak for the carbon electrode with nanoparticles is shifted to the left, to more negative values, relative to the Au electrode, which corresponds to higher energy for the reduction reaction.

Besides the fact that the planar Au electrode could facilitate the reduction of the species, the above results showing higher currents densities, lower reduction potentials and narrower width are a testimony that the enzyme is catalyzing in a more appropriate environment. That is, the catalytic site of the enzyme is requiring a lower and restricted energy, which was only achieved when the enzyme is sitting on lipids.

There are many biosensors in the literature developed from modified electrodes with different SAMs, enzymes and lipid bilayers composition (Huang et al., 2003; Liu et al., 2006; Wang et al., 2008). It is known that the method chosen for the immobilization of an element of recognition interferes with the biosensor performance parameters such as sensitivity, selectivity and mainly stability, which in biosensors in general can be a considerable problem. The disadvantage of the present sensor is the fragility and the limited stability of the supported lipid bilayers in a certain period of time. Also, electrochemistry was used as a detection method, which is based on redox reactions. This approach would not be interesting to use in biological samples because of vitamin C interference. The concentrations of the main detection interferents, ascorbic acid (AA), are usually several orders of magnitude higher (ascorbic acid levels are generally within 0.1–0.6 mM; Robinson et al., 2008). However, the present system is suitable as one-shot sensor for the rapid detection of dopamine on prepared samples or pharmaceutical products. Also, the developed sensor, is cheap, easy to handle and can be prepared easily in the laboratory. Additionally, the HRP-lipid bilayer system gives a satisfactory detection limit value when compared with some others sensors developed for dopamine determination described in the literature (Caruso et al., 1999; Fritzen-Garcia et al., 2009a; Kim et al., 2010; Leite et al., 2003; Lupetti et al., 2005; Raoof et al., 2010).

Conclusions

The lipid bilayers may provide a natural environment for the immobilization of bioactive molecules such as enzymes, antibodies, and antigens since the phospholipids mimic the cellular membranes. The present results indicate that HRP immobilized on lipid bilayers of DMPC can catalyze the redox reaction of dopamine efficiently, given electrochemical responses with intense and narrow current peaks, low background and at low potential values. The AFM images showed that the enzyme is adsorbed at the external layer of the lipid bilayer, with the formation of protrusions on the surface and confirmed by the distribution of the gold particles in the images after sample immunolabeling procedure. Although the electrical charges on the surface were not measured, the enzyme and phospholipids surface interaction occurs probably by electrostatic forces due to the pH used in the experiments. The biosensor based on HRP immobilized DMPC bilayers supported on Au (111) by DTT self-assembled monolayer was successful as one-shot sensor for the rapid detection of dopamine with a higher sensitivity, lower background and reduction potentials, higher currents densities and narrower width if compared with the sensor developed in our previous work. Although, our goal was not to develop a method to measure dopamine concentration in biological fluids, since HRP is not specific for dopamine, our detection system can be beneficial giving some insights in this issue. Moreover, the electrodes may be

useful to titrate pharmaceutical formulations of dopamine as well as in fundamental studies to further investigation of interactions between different micro-and macromolecules and lipid membranes, as well as in the development of new technology for nanostructured materials.

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