Available online at www.sciencedirect.com
ScienceDirect
Electrochemistry Communications 8 (2006) 1665–1670
electrochemistry communications
www.elsevier.com/locate/elecom

A strategy for enzyme immobilization on layer-by-layer dendrimer–gold nanoparticle electrocatalytic membrane incorporating redox mediator

Frank N. Crespilho [a,b], M. Emilia Ghica [b], Monica Florescu [c], Francisco C. Nart [a], Osvaldo N. Oliveira Jr. [d], Christopher M.A. Brett [b,*]

[a] *Instituto de Química de São Carlos, Universidade de São Paulo, 13560-970, Brazil*
[b] *Departamento de Química, Universidade de Coimbra, 3004-535 Coimbra, Portugal*
[c] *Department of Physics, Transilvania University of Brasov, Romania*
[d] *Instituto de Física de São Carlos, Universidade de São Paulo, 13560-970, Brazil*

Received 5 July 2006; received in revised form 21 July 2006; accepted 24 July 2006
Available online 28 August 2006

Abstract

A new approach is described to produce nanostructured electrocatalytic membranes using a combination of three methods. Using the layer-by-layer technique, PAMAM dendrimers with cobalt hexacyanoferrates-modified gold nanoparticles were alternated with poly(vinylsulfonic acid) layers on ITO (indium tin oxide) electrodes. This film was used as substrate for immobilization of glucose oxidase in the presence of bovine serum albumin and glutaraldehyde as cross-linker. The modified electrode was successfully applied as a biosensor for the amperometric measurement of glucose, using glucose oxidase enzyme, at 0.0 V vs. SCE.

Keywords: Layer-by-layer; PAMAM dendrimers; Gold nanoparticles; Enzyme immobilization; Redox mediator; Biosensor

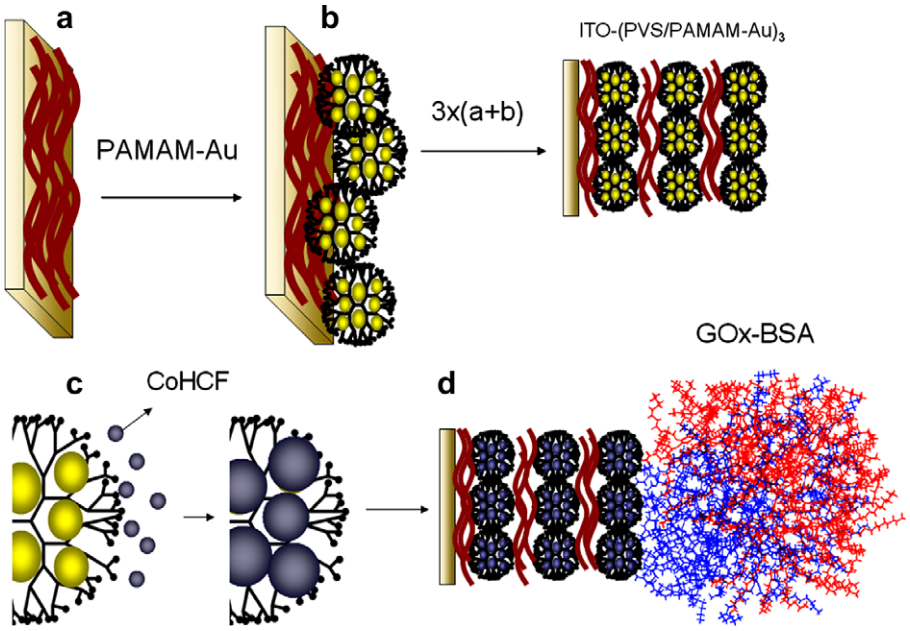
1. Introduction

Surface enzyme immobilization has become an important topic in nanodevices [1–6], especially for biosensing where the main challenge is to combine the features required for self-sufficient operation in the same electrode. Such an ideal biosensor has to fulfil various criteria, including electrochemical reversibility, low overpotential and high selectivity. Furthermore, a friendly environment is necessary to immobilize enzymes in hybrid nanomaterials with optimized preservation of enzyme activity. Several approaches have been used to produce electrode assemblies, among which the layer-by-layer (LbL) [1] deposition method has given excellent results. It allows control of the molecular architecture and suitable choice of template materials for enzyme immobilization, as is the case of poly(amidoamine) dendrimers (PAMAM) [2]. In biosensors involving the electrode reaction of hydrogen peroxide, attempts have been made to catalyze this reaction with an oxidase enzyme at a less positive potential. The aim is to inhibit direct oxidation responses to interferents, such as ascorbic acid and uric acid. For example, Yang et al. [3] studied multilayer films of glucose oxidase (GOx/Au-nanoparticles) on an Au electrode surface using cysteamine as a covalent attachment cross-linker. However, the main disadvantage of the method is the addition of the redox mediator to the reaction medium, which is less efficient than using an immobilized redox mediator.

In this communication we show a new strategy (Scheme 1) to develop an enzyme biosensor, based on three principles: (i) preparation of a membrane-substrate with high capability for hydrogen peroxide diffusion, (ii) use of an efficient redox mediator for electrocatalytic reduction of

* Corresponding author. Tel.: +351 239 835295.
E-mail address: brett@ci.uc.pt (C.M.A. Brett).

1388-2481/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.elecom.2006.07.032



Scheme 1. Schematic fabrication of LbL films comprising PVS and PAMAM–Au. The sequential deposition of LbL multilayers was carried out by immersing the substrates alternately into PVS (a) and PAMAM–Au (b) solutions for 5 min per step. After deposition of 3 bilayers, an ITO–(PVS/PAMAM–Au)$_3$@CoHCF electrode was prepared by potential cycling (c) The enzyme immobilization to produce ITO–(PVS/PAMAM–Au)$_3$@CoHCF-GOx (d) was carried out in a solution containing BSA, glutaraldehyde and GOx.

hydrogen peroxide at 0.0 V (vs. SCE), where oxidation of most electrochemical interferents is avoided and (iii) a hybrid membrane-mediator which is environmentally friendly for enzyme immobilization. Metal hexacyanoferrate films have been shown to be efficient redox mediators for electrochemical enzyme biosensors [4]. They are mixed-valence clusters that can transfer electrons during reduction and oxidation processes. In particular, cobalt hexacyanoferrate (CoHCF) films have been characterized and investigated for different purposes [5,7]. This concept of depositing a redox mediator around gold nanoparticles was applied in our previous work using Prussian Blue on the shell of gold nanoparticles [8].

With the combination of three techniques, LbL, electrodeposition and cross-linking, molecules of GOx have been immobilized at a modified ITO (indium tin oxide) electrode.

2. Experimental

2.1. Reagents and buffers

Glucose oxidase (GOx, E.C. 1.1.3.4, from *Aspergillus niger*, 24 U/mg) was from Fluka. α-D(+)-glucose, glutaraldehyde (GA) (25% v/v) and bovine serum albumin (BSA) were purchased from Sigma. Hydrogen peroxide (H_2O_2) 35% was from José M. Vaz Pereira. Potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) and cobalt chloride ($CoCl_2 \cdot 6H_2O$) were obtained from Merck. For electrochemical experiments: cyclic voltammetry and amperometry, the supporting electrolyte was sodium phosphate buffer saline (NaPBS) (0.1 M $NaH_2PO_4/Na_2HPO_4 + 0.05$ mol L^{-1} NaCl, pH 7.0). Stock solutions of 1 mol L^{-1} glucose and 100 mmol L^{-1} hydrogen peroxide were prepared in supporting electrolyte and were kept in the refrigerator. Hydrogen peroxide solutions were standardized by titration with acidified $KMnO_4$ solution. All chemicals were of analytical grade and used without further purification. All solutions were prepared with Millipore Milli-Q nanopure water (resistivity >18 MΩ cm).

2.2. PAMAM–Au synthesis

Nanohybrids were prepared as follows [8]: 2 mL of $KAuCl_4$ solution (1 mmol L^{-1}) were added to 2 mL of G4 PAMAM (0.07 mmol L^{-1}) and 2 mL of formic acid (1 mmol L^{-1}). This solution was vigorously stirred for 2 min. The nanoparticle growth kinetics was followed by UV–Vis spectrophotometry (Fig. 1), using a Hitachi U-2001 Spectrophotometer, USA. The morphology and particle size distribution were characterized using a 200 kV transmission electron microscope (TEM, Model CM200; Philips, Netherlands).

2.3. PVS/PAMAM–Au multilayer self-assembly

LbL films were assembled onto ITO-coated glass [8–10]. The concentration of the dipping solutions was set at 0.07 mmol L^{-1} and 0.5 g L^{-1} for PAMAM–Au and PVS, respectively. The sequential deposition of multilayers was carried out by immersing the substrates alternately into the PAMAM–Au and PVS solutions for 5 min [1]. After the deposition of each layer, the substrate/film system was rinsed and dried in a flow of N_2 gas.

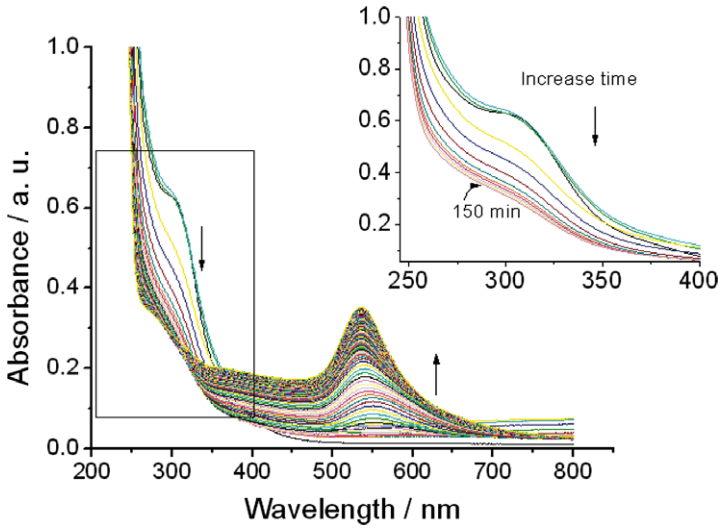


Fig. 1. Electronic spectra (UV–Vis) at different time intervals showing gold nanoparticle formation. The spectra were recorded at 10 min intervals. Total experiment time: 1500 min.

2.4. Electrochemical measurements

Measurements were performed in a one-compartment cell of 15 mL volume containing the ITO modified electrode (1.0 cm^2), a platinum auxiliary electrode and a saturated calomel electrode (SCE) as reference. Voltammetric and amperometric experiments were carried out using a CV-50 W Voltammetric Analyzer from Bioanalytical Systems, West Lafayette, IN, USA, controlled by BAS CV-2.1 software. The pH measurements were carried out with a CRISON 2001 micro pH-meter. All measurements were done at room temperature (25 ± 1 °C).

2.5. Electrode preparation and enzyme immobilization

To prepare the optimised electrode assembly, three bilayers of (PVS/PAMAM–Au) were deposited onto the ITO electrode, then CoHCF film was deposited and finally GOx was immobilized by a cross-linking procedure. The cobalt hexacyanoferrate [5] was electrochemically deposited by cycling the electrode 30 times between −0.2 and 0.9 V vs. SCE at a scan rate of 50 mV s^{-1} in a freshly prepared solution containing 0.5 mmol L^{-1} $CoCl_2$, 0.25 mmol L^{-1} $K_3Fe(CN)_6$ and 0.05 mol L^{-1} NaCl at pH 3 (pH adjusted with HCl) under slow stirring conditions. Afterwards the CoHCF-modified electrode was stabilized for 1 h in 0.05 mol L^{-1} NaCl, pH 3. GOx was immobilized using a cross-linking procedure from a mixture of glutaraldehyde, enzyme and bovine serum albumin. To prepare 340 μL of this enzyme mixture, 100 μL of GA (2.5% v/v diluted in water) were mixed with 240 μL enzyme solution. The enzyme solution was prepared by dissolving 20 mg BSA and 50 mg GOx in 1 mL of 0.1 mol L^{-1} NaPBS (pH 7). CoHCF-modified electrodes were immersed in this mixture for 2 h and then allowed to dry at room temperature for 1 h.

3. Results and discussion

First, Au nanoparticles were grown inside PAMAM molecules in aqueous solution using formic acid as the reducing agent [8]. This solution has a yellow pale colour – when the zerovalent Au complex is formed the colour immediately changes from yellow to red, which can be followed by changes in the UV–Vis absorption spectra. This reaction occurred over a 4 h time period. The initial reduction stage is shown by the decrease in absorption of the Au (III) band at 300 nm, see Fig. 1, and the peak that appears at around 500 nm is associated with the plasmon resonance, corresponding to nanoparticle growth. Results obtained by TEM after 200 min reaction showed well-organized Au nanoparticles (Fig. 2), with a particle diameter of approximately 3 nm and a narrow size distribution. The particle size distribution was estimated by the measurement of at least 200 particles in TEM images. In addition, X-ray diffraction of PAMAM–Au cast films (not shown) enabled easy identification of the (111), (200) and (220) atomic planes of the Au nanoparticles.

The solution containing the PAMAM–Au nanoparticles, obtained by this synthesis, was used as cationic polyelectrolyte to assemble a 3-bilayer PVS/PAMAM–Au film onto the ITO electrode, where PVS (poly(vinylsulfonic acid)) was used as the anionic polyelectrolyte by the LbL technique [1,8–10]. This was followed by electrodeposition by potential cycling of cobalt hexacyanoferrate (CoHCF) around the Au nanoparticles, as shown in Fig. 3a, leading to what will be referred to as an electrocatalytic membrane.

The resulting CoHCF electrocatalytic membrane, ITO-$(PVS/PAMAM–Au)_3$@CoHCF, was characterized in phosphate buffer solution (pH 7.0), the electrolyte commonly used for enzyme substrate measurements. The electrode was cycled at different scan rates, and the Co redox peaks were well defined. The current peaks increased line-

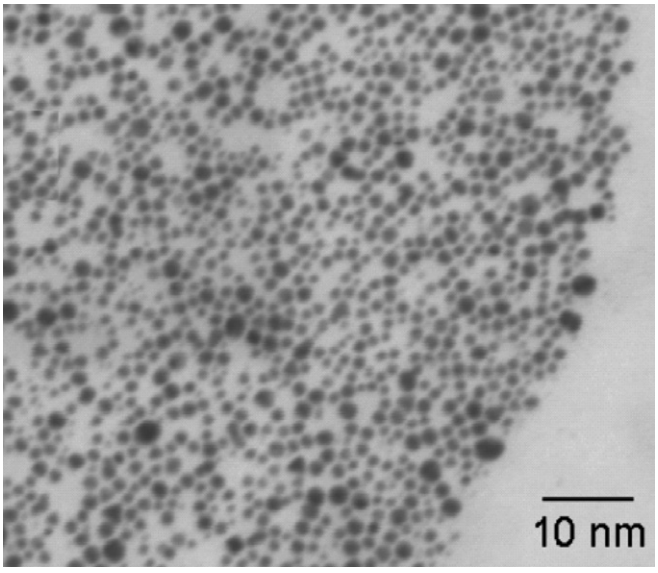


Fig. 2. Transmission electron microscopy (TEM) of PAMAM–Au using a copper-grid covered by a polymeric carbon film. The average size of nanoparticles was 3 nm.

arly with scan rate up to 300 mV s^{-1}, indicating mediator immobilization and fast charge transport (Fig. 3b). Cyclic voltammograms of ITO–(PVS/PAMAM–Au)$_3$@CoHCF in the same solution with and without addition of 0.5 mM hydrogen peroxide are shown in Fig. 3c. The height of both anodic and cathodic peaks decreased with addition of hydrogen peroxide. At this pH electrocatalytic reduction of hydrogen peroxide can occur with formation of hydroxyl ions [5], which leads to a decrease in the height of the CoHCF peaks.

Glucose oxidase (GOx) was immobilized on the PVS/PAMAM–Au@CoHCF electrocatalytic membrane, by a cross-linking procedure using a mixture of glutaraldehyde (GA), enzyme and bovine serum albumin (BSA). GA is a bifunctional cross-linking agent which reacts with lysine residues on the exterior of the proteins; addition of bovine serum albumin accelerates the cross-linking process due to the lysine groups present in its structure [11]. This approach leads to more efficient catalysis than with direct enzyme immobilization, probably because biological activity losses are prevented owing to the friendly environment for enzyme immobilization. With BSA molecules the enzyme maintains its catalytic sites more accessible for redox reactions, since cross-linking does not affect the GOx molecules significantly so there can be higher enzyme activity and greater stability. Previous work using different enzymes and substrates e.g. [12–14] showed that in the presence of BSA the biosensor response is improved.

Three different strategies were evaluated to optimise the biosensor construction, based on use of three bilayers of dendrimer containing gold nanoparticles alternately with PVS. The strategies used were (a) applying PVS/PAMAM–Au nanoparticle bilayer, mediator and enzyme layer three times, i.e. (PVS/PAMAM–Au@CoHCF@-GOx)$_3$, (b) applying PVS/PAMAM-Au nanoparticles and depositing mediator three times followed by the enzyme, i.e. (PVS/PAMAM–Au@CoHCF)$_3$–GOx, and finally (c)

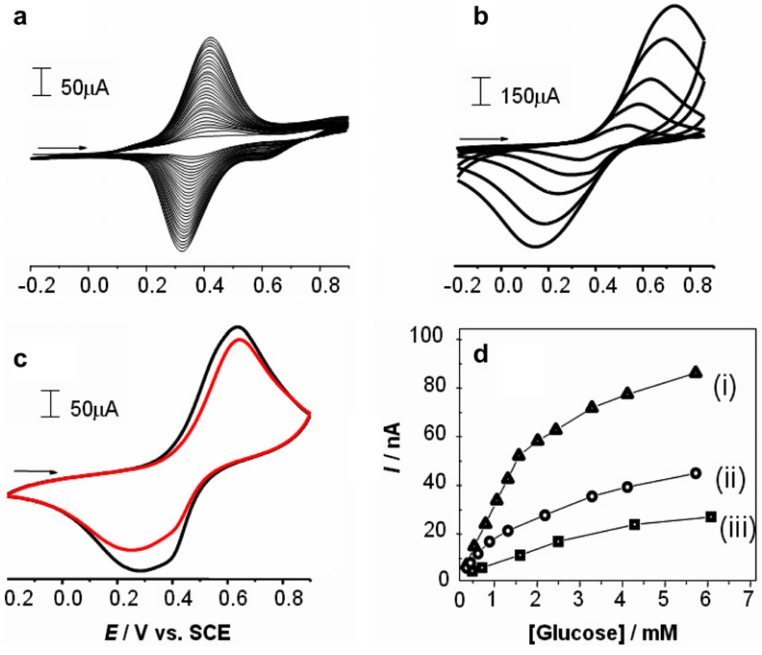
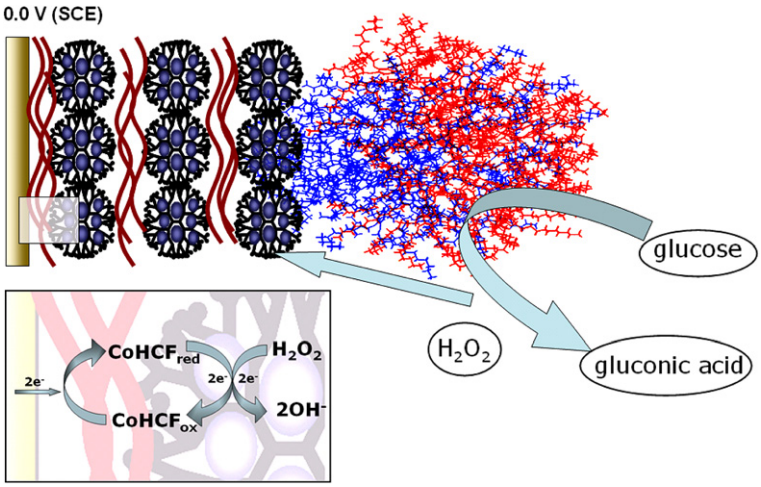


Fig. 3. (a) Cyclic voltammograms showing continuous growth of CoHCF on ITO–(PVS/PAMAM-Au)$_3$ electrode. Scan rate 50 mV s^{-1}. (b) Cyclic voltammograms of ITO-(PVS/PAMAM-Au)$_3$@CoHCF in 0.1 M NaPBS (pH 7.0) at different scan rates (from inner curve to outer curve: 20, 50, 100, 200, 300 mV s^{-1}). (c) Cyclic voltammograms of the ITO-(PVS/PAMAM-Au)$_3$@CoHCF in 0.1 mol L^{-1} NaPBS (pH 7.0) without (black line) and with (red line) addition of 0.5 mM hydrogen peroxide. Scan rate 50 mV s^{-1}. (d) Calibration curves for glucose at 0.0 V using different strategies: (i) (PVS/PAMAM-Au)$_3$@CoHCF-GOx, (ii) (PVS/PAMAM-Au@CoHCF)$_3$-GOx and (iii) (PVS/PAMAM-Au@CoHCF@GOx)$_3$.



Scheme 2. Schematic representation of reaction of glucose at ITO–(PVS/PAMAM–Au)$_3$@CoHCF–GOx electrode.

applying PVS/PAMAM–Au nanoparticles three times, then depositing mediator followed by enzyme, i.e. (PVS/PAMAM–Au)$_3$@CoHCF–GOx. Calibration curves for glucose using these three strategies are presented in Fig. 3d. It can be observed that in all cases the response of the biosensor to glucose has a Michaelis–Menten like behaviour. Comparing the three curves it is clear that the biosensor response is greater when the enzyme is immobilized on the top (curves (i) and (ii)) than when it was immobilized with each gold nanoparticle/mediator layer (curve (iii)). The best response to glucose was obtained with the last configuration, i.e. ITO–(PVS/PAMAM–Au)$_3$@CoHCF-GOx. So, for further experiments this electrode configuration was used, see Scheme 2.

In order to study the influence of glucose addition on the electrode response, the biosensor was characterised by cyclic voltammetry. Cyclic voltammograms recorded in buffer and with addition of glucose led to a decrease in both oxidation and reduction peaks of CoHCF, similar to that shown in Fig. 3c. This is indicative of electrocatalytic reduction of hydrogen peroxide on PAMAM–Au@CoHCF, as discussed above.

Based on previous work with CoHCF modified carbon film electrodes and GOx [5], the evaluation of the amperometric response of the biosensor was performed at 0.0 V vs. SCE. The biosensor showed a sensitivity of 33.6 ± 0.2 nA mmol L^{-1} cm^{-2} and the detection limit (three times the signal-to-noise ratio) was 17 μmol L^{-1} (Fig. 3d (i)). The apparent Michaelis–Menten constant determined from the Lineweaver–Burk plot was 2.03 mM. With the new biosensor the linear range was up to 1.5 mmol L^{-1} whilst in the same conditions with carbon film electrodes and CoHCF redox mediator the linear range for glucose determination was up to 30 μmol L^{-1} (detection limit 4 μmol L^{-1}) [5]. This represents better characteristics for most bioanalytical applications where a wider concentration range is usually required in order to avoid complicated dilution steps.

4. Conclusions

An electrochemical enzyme biosensor with glucose oxidase immobilized at PVS/PAMAM-Au@CoHCF electroactive membrane has been developed. Using CoHCF as redox mediator, hydrogen peroxide (the enzymatic reaction product) was determined at 0.0 V (vs. SCE). This applied potential ensures minimization of interference effects when the biosensor is used in real and complex matrices, such as biological media, food and beverages. The measurements, carried out with hydrogen peroxide and glucose, demonstrate that this new approach is extremely promising for the construction of enzyme biosensors.

Acknowledgements

Financial support from FAPESP, CAPES (Process No. 1238/05-1), CNPq, IMMP/MCT (Brazil), European Project HPRN-CT-2002-00186, Fundação para a Ciência e Tecnologia (FCT) Portugal (SFRH/BD/14014/2003 – MEG) and ICEMS (Research Unit 103), is gratefully acknowledged.

References

[1] G. Decher, Science 277 (1997) 1232.
[2] H.C. Yoon, M.Y. Hong, H.S. Kim, Anal. Chem. 72 (2000) 4420.
[3] W. Yang, J. Wang, S. Zhao, Y. Sun, C. Sun, Electrochem. Commun. 8 (2006) 665.
[4] F. Ricci, G. Palleschi, Biosens. Bioelectron. 21 (2005) 389.
[5] M. Florescu, C.M.A. Brett, Anal. Lett. 37 (2004) 871.
[6] J.A. Hansen, J. Wang, A.N. Kawde, Y. Xiang, K.V. Gothelf, G. Collins, J. Am. Chem. Soc. 128 (2006) 228.
[7] N.R. de Tacconi, K. Rajeshwar, R.O. Lezna, Chem. Mater. 15 (2003) 3046.
[8] F.N. Crespilho, V. Zucolotto, C.M.A. Brett, O.N. Oliveira Jr., F.C. Nart, J. Phys. Chem. B (in press).
[9] F.N. Crespilho, V. Zucolotto, J.R. Siqueira Jr., C.J.L. Constantino, F.C. Nart, O.N. Oliveira Jr., Environ. Sci. Technol. 39 (2005) 5385.
[10] F.N. Crespilho, F. Huguenin, V. Zucolotto, P. Olivi, F.C. Nart, O.N. Oliveira Jr., Electrochem. Commun. 8 (2006) 348.

[11] C.J.S.M. Silva, F. Sousa, G. Gübitz, A. Cavaco-Paulo, Food Technol. Biotechnol. 42 (2004) 56.
[12] S. Milardovic, Z. Grabaric, B.S. Grabaric, Food Technol. Biotechnol. 38 (2000) 2003.
[13] S. de Luca, M. Florescu, M.E. Ghica, A. Lupu, G. Palleschi, C.M.A. Brett, D. Compagnone, Talanta 68 (2005) 171.
[14] P.A. Fiorito, C.M.A. Brett, S.I.C. de Torresi, Talanta 69 (2006) 403.