Available online at www.sciencedirect.com
SCIENCE DIRECT®
Talanta 64 (2004) 115–120
Talanta
www.elsevier.com/locate/talanta

Electrochemistry and scanning electron microscopy of polyaniline/peroxidase-based biosensor

Ntlatseng G.R. Mathebe, Aoife Morrin, Emmanuel I. Iwuoha*

Department of Chemistry, University of the Western Cape, P/Bag X17, Bellville 7535, South Africa

Received 12 August 2003; received in revised form 11 November 2003; accepted 19 November 2003

Available online 19 May 2004

Abstract

An amperometric biosensor was prepared by in situ deposition of horseradish peroxidase (HRP) enzyme on a polyaniline (PANI)-doped platinum disk electrode. The PANI film was electrochemically deposited on the electrode at 100 mV s^{-1}/Ag–AgCl. Cyclic voltammetric characterization of the PANI film in 1 M HCl showed two distinct redox peaks, which prove that the PANI film was electroactive and exhibited fast reversible electrochemistry. The surface concentration and film thickness of the adsorbed electroactive species was estimated to be 1.85×10^{-7} mol cm^{-2} and approximately 16 nm, respectively. HRP was electrostatically immobilized onto the surface of the PANI film, and voltammetry was used to monitor the electrocatalytic reduction of hydrogen peroxide under diffusion-controlled conditions. Linear responses over the concentration range 2.5×10^{-4} to 5×10^{-3} M were observed. Spectroelectrochemistry was used to monitor the changes in UV–vis properties of HRP, before and after the catalysis of H_2O_2. The biosensor surface morphology was characterized by scanning electron microscopy (SEM) using PANI-doped screen-printed carbon electrodes (SPCEs) in the presence and absence of (i) peroxidase and (ii) peroxide. The SEM images showed clear modifications of the conducting film surface structure when doped with HRP, as well as the effect of hydrogen peroxide on the morphology of biosensor.

Keywords: Amperometric biosensor; Conducting polymers; Polyaniline-based biosensor; Scanning electron microscopy of biosensor; Peroxidase-based biosensor

1. Introduction

Organic conducting polymers have attracted interest in recent years because they exhibit a wide range of novel electrochemical properties [1]. Among the most studied is polyaniline (PANI), which has been studied extensively as an important conducting material that possesses interesting electrical, electrochemical, and optical properties [2]. The continuous growing interest in the study of PANI is caused by these diverse, unique properties, and its promising potential in commercial applications. The potential applications of PANI include anti-corrosive coatings [3,4], secondary batteries [5], electrochromic devices [6], and electrochemical biosensors [7–10].

Advantages of utilizing polyaniline-coated electrodes in biosensors are impressive signal amplification and elimination of electrode fouling. This polymer also provides a suitable environment for immobilization of biomolecules. Polymer films can be deposited on electrode surfaces very readily. Polymerization of the monomer aniline, can be achieved either chemically or electrochemically. Electrochemically, the polymer can be grown on the electrode surface by pulse [11,12], galvanostatic [11], potentiostatic [13] or potentiodynamic [8,9,13–15] techniques. The latter being preferred because of the homogenous film produced [8] and a strong adherence to the electrode surface [11]. An important advantage of the electropolymerization technique in the fabrication of a biosensor is that the film thickness and characteristics can be controlled by monitoring polymerization charge [16].

Determination of hydrogen peroxide and other organic peroxides is of practical importance in clinical, environmental, and many other fields. Hydrogen peroxide has been determined by volumetric, colorimetric, and chemiluminescence methods that tend to be complex, time-consuming, and suffer from various interferences. The sensitive

* Corresponding author. Tel.: +27-21-959-3054; fax: +27-21-959-2030.
E-mail address: eiwuoha@uwc.ac.za (E.I. Iwuoha).

0039-9140/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2003.11.050

determination of hydrogen peroxide may be achieved by the use of peroxidase-modified electrodes [17], since enzymes are known to show excellent selectivity for their substrates. The fabrication of H_2O_2 sensors has attracted great interest as the measurement of H_2O_2 is the basis of detecting many biologically active materials such as glucose and cholesterol.

This paper reports the fabrication and characterization of a polyaniline-based biosensor incorporating the enzyme horseradish peroxidase (HRP) for the detection of hydrogen peroxide. Both square wave and cyclic voltammetry have been used as detection methods for hydrogen peroxide. The sensor was interrogated using electrochemistry, spectroelectrochemistry (UV–vis) and scanning electron microscopy (SEM) as characterization techniques.

2. Experimental

2.1. Reagents

All reagents were purchased from Sigma–Aldrich (Cape Town, South Africa) and were of analytical grade. The enzyme peroxidase (EC 1.11.1.7 type II from horseradish, 150–250 units mg^{-1}) was used for biosensor preparation. One unit of HRP will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6 at 20 °C. Aniline was distilled before use, and fresh solutions of hydrogen peroxide were prepared daily. Phosphate buffer solution (0.05 M, pH 6.8) was prepared from anhydrous disodium hydrogen phosphate and potassium dihydrogen phosphate monohydrate. The anhydrous salt was dried for 2 h at 110 °C and cooled in a desiccator before being used for buffer preparation. Analytical grade argon (Afrox, SA) was used to degas the system.

2.2. Apparatus

All electrochemical experiments were carried out and recorded with a computer interfaced to a BAS/50W integrated automated electrochemical workstation (Bioanalytical Systems, Lafayette, IN, USA). Cyclic voltammetry, square wave voltammetry and differential pulse voltammetry were carried out in a 20 ml electrochemical cell, with Ag/AgCl (3 M NaCl type) and platinum wire mesh as reference and auxiliary electrodes, respectively. A Faraday cage (BAS C2) was used for all experiments. A platinum disk electrode and screen-printed carbon electrodes (SPCEs) were used as working electrodes. The Pt disk (1.77×10^{-2} cm^2) was obtained from BAS. The screen-printed electrodes (0.09 cm^2) were donated by Dublin City University, Ireland, and their fabrication is described elsewhere [18]. Alumina micropolish and polishing pads (Buehler, IL, USA) were used for electrode polishing. UV–vis absorbance measurements were recorded at room temperature on a UV/VIS 920 spectrometer (GBC Scientific Instruments, Australia) using a specially designed quartz cell (designed in-house), incorporating a Pt gauze, Ag/AgCl, and Pt wire as working, reference, and auxiliary electrodes, respectively. Scanning electron microscopy was performed with a Hitachi X650 scanning electron microscope. An accelerating voltage of 15 kV was employed.

2.3. Polymerization of aniline

The Pt disk electrode was first cleaned by polishing with 1, 0.3, and 0.05 μm alumina, respectively, and then rinsed thoroughly with de-ionized water after each polishing step. Both the Pt disk and screen-printed electrodes were subjected to repeated potential scanning in 0.2 M H_2SO_4 in the potential range of −1200 to +1500 mV until the voltammograms obtained were reproducible. Polymerization was achieved in a potentiodynamic mode in a 0.2 M aniline per 1 M HCl solution. The potential was cycled between −200 and +1100 mV at a scan rate of 100 mV s^{-1} for 10 cycles.

2.4. Enzyme immobilization

The Pt/PANI electrode was first reduced in 10 ml of 0.05 M phosphate buffer solution, pH 6.8 at −500 mV for 15 min until a steady state was achieved. Enzyme immobilization was achieved by oxidation of the PANI film in the presence of 20 μl HRP (4 mg ml^{-1}) at a potential of +650 mV for 20 min. During the oxidation process, HRP became electrostatically attached to the polymer film. The Pt/PANI/HRP electrode was rinsed with de-ionized water to remove any loosely bound enzyme, and stored in buffer solution (pH 6.8) at 4 °C when not in use.

2.5. Electrochemical measurements

The cell used for the electrocatalytic reduction of hydrogen peroxide consisted of Pt/PANI/HRP electrode, platinum wire mesh, and Ag/AgCl as the working, counter, and reference electrode, respectively. The 10 ml test solution containing 0.05 M phosphate buffer (pH 6.8) was degassed with argon after each addition of small amounts of 0.1 M H_2O_2. The concentration range of peroxide studied was between 2.5×10^{-4} and 5×10^{-3} M. Square wave and cyclic voltammetry were used to measure the responses of the biosensor to H_2O_2. Square wave voltammetry was carried out using a step potential of 4 mV, a frequency of 5 Hz, and amplitude of 50 mV. All cyclic voltammograms (CVs) were performed at 5 mV s^{-1}.

3. Results and discussion

3.1. Electrosynthesis of PANI on platinum

Polyaniline was electrochemically synthesized from an acidic medium of aniline by sweeping the potential from −200 to +1100 mV at 100 mV s^{-1}. Fig. 1 shows a typical cyclic voltammogram of Pt in 0.2 M aniline per 1 M HCl

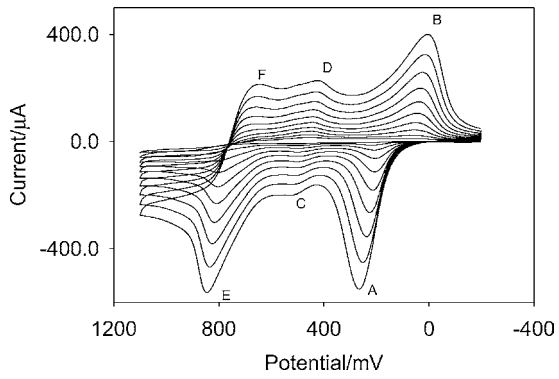

Fig. 1. Electrosynthesis of the PANI film in HCl (1 M) on a Pt disk electrode surface (area: 1.77×10^{-2} cm^2). Successive polymerization cycles are shown which correspond to increases in the thickness of the film during deposition.

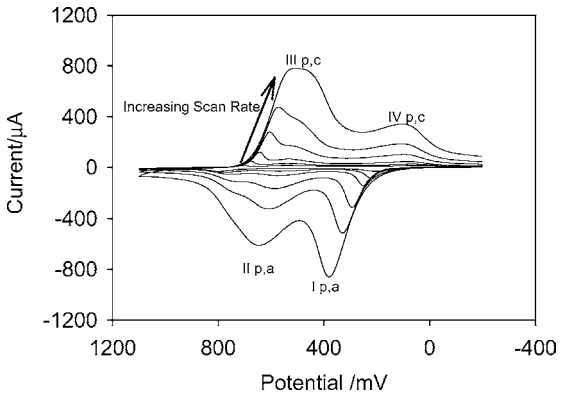

Fig. 2. Multi-scan CV of Pt/PANI in 1 M HCl. The peaks were assigned as follows: peaks $I_{p,a}/IV_{p,c}$: polyleucoemeraldine radical cation /polyleucoemeraldine. Peaks $II_{p,a}/III_{p,c}$: polyemeraldine/polyemeraldine radical cation. Scan rates: 5, 10, 20, 50, 100, and 200 mV s^{-1}.

solution for this electropolymerization process. The PANI layer was seen to be redox active in the potential region studied, exhibiting three sets of redox peaks. Initially, oxidation of aniline occurred at approximately +900 mV resulting in the nucleation of PANI. During subsequent scans, the oxidation of aniline occurred at lower potentials due to the catalytic effect of PANI, which resulted in deposition of polymer on the electrode surface. Redox couples A/B and E/F were attributed to intrinsic redox processes of the polymer itself. The redox couple A/B occurred at approximately +200 mV and is attributed to the transformation of PANI from the reduced leucoemeraldine (LE) state to the partly oxidized emeraldine state (EM). The redox couple E/F at approximately +800 mV corresponds to transition of the PANI from LE to pernigraniline (PE) state, and is accompanied by the oxidation of aniline monomer [15]. The redox couple C/D at approximately +500 mV, which is generally attributed to the redox reaction of *p*-benzoquinone, [14,15] is less intense.

An increase in the amplitude of the redox peaks was observed as a consequence of repeated potential scans, indicative of polymer deposition at the Pt surface and confirmed that the polymer was conducting. This electrosynthesized PANI film served two purposes when applied in this biosensor. It behaved as an electron mediator, shuttling electrons between the immobilized enzyme and the electrode surface, and also served as a point of attachment for the protein (HRP) [9].

3.2. CV characterization of Pt/PANI electrode in HCl (1 M)

Multi-scan rate voltammograms of the PANI-modified Pt electrode in HCl (1 M) solution are shown in Fig. 2. Both peak potentials and corresponding peak currents varied as the scan rate values varied, which showed that the polymer was electroactive and diffusion of electrons was taking place along the polymer chain. The linear dependence of peak currents of anodic peak *I* on the scan rate indicated that we have a thin film of surface-bound conducting electroactive polymer, undergoing rapid reversible electron transfer reaction. The surface concentration of the PANI film, Γ^*_{PANI} can be estimated from a plot of I_p against υ in accordance with Brown–Anson model [19] using the equation:

$$I_p = \frac{n^2 F^2 \Gamma^*_{PANI} A \upsilon}{4RT} \quad (1)$$

where n represents number of electrons transferred (2), F is the Faraday constant (96,584 C mol^{-1}), Γ^*_{PANI} is the surface concentration of PANI film (mol cm^{-2}), A is the surface area of the electrode (0.0177 cm^2), υ is the scan rate (V s^{-1}), R is the gas constant (8.314 J (mol K)$^{-1}$), and T is the absolute temperature of the system (298 K).

The surface concentration of PANI film, Γ^*_{PANI}, was estimated to be 1.85×10^{-7} mol cm^{-2}. On increasing the scan rate, the magnitude of the peak current increased. However, the peak potentials of all the four peaks shifted to more positive potentials and became broad. This shows that the peak currents are diffusion-controlled. Thus, the Randel–Sevćik equation was used to determine the rate of electron transport (i.e., diffusion coefficient of the electrons, D_e) within the polymer. Using the Randel–Sevćik equation, D_e was 8.68×10^{-9} cm^2 s^{-1}. Iwuoha et al. reported a D_e value of 6.46×10^{-8} cm^2 s^{-1} for a PANI film doped with polyvinyl sulphonate (PVS). Since D_e depends on the electro-deposition conditions used and the homogeneity of the film, inclusion of the ionic co-polymer polyvinyl sulphonate by Iwuoha et al. increased the conductivity of the PANI film, resulting in a D_e value approximately one order of magnitude higher than that reported here.

3.3. SWV characterization of Pt/PANI/HRP electrode in phosphate buffer, pH 6.8

Square wave voltammetry was used to examine the peak potentials of the Pt/PANI/HRP in phosphate buffer, pH 6.8. Fig. 3 represents the net (difference between forward and reverse currents) SWV responses when the Pt/PANI/HRP

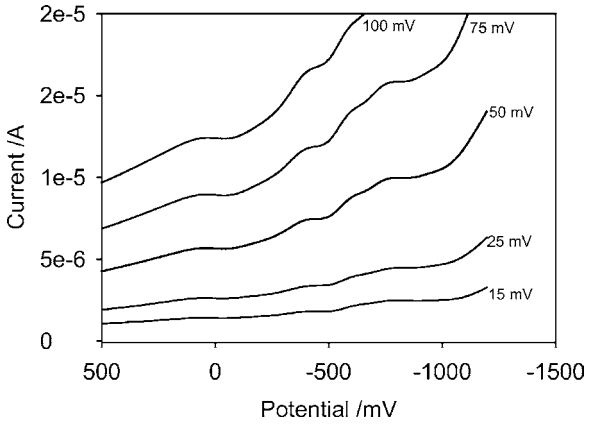

Fig. 3. Net cathodic square wave voltammograms of the Pt/PANI/HRP electrode performed at 15, 25, 50, 75, and 100 mV. Experimental conditions were: frequency, 30 Hz and potential step, 4 mV.

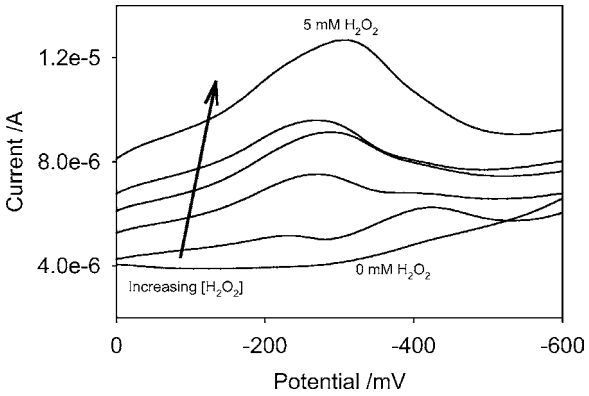

Fig. 4. Net cathodic square wave voltammograms of the Pt/PANI/HRP electrode in the presence of different concentrations of H_2O_2. Experimental conditions were: frequency, 5 Hz; amplitude, 50 mV; and potential step, 4 mV.

electrode was scanned reductively over a range of amplitudes from 15 to 100 mV at a frequency of 30 Hz. A prominent peak at −400 mV was observed and exploited to monitor electrocatalytic reduction of H_2O_2 at a Pt/PANI/HRP electrode. Increasing the amplitude resulted in increased currents of this peak. However, increased amplitude was also associated with higher background currents. Therefore, amplitude of 50 mV was taken for future work as a compromise between these parameters.

3.4. PANI-mediated electrocatalytic reduction of H_2O_2

HRP was immobilized onto the surface of the polymer by electrostatic interactions with the polymer backbone by application of a potential of +650 mV in the presence of HRP for a period of time (Section 2.4). This effective biosensor format facilitated electron transfer between the enzyme and the electrode. Without the incorporation of a mediator, such as PANI, electron transfer between the redox center of the enzyme and the electrode is very slow [20]. This paper demonstrates facilitated electron transfer between the platinum electrode and the enzyme HRP by the electroactive polymer, PANI, in phosphate buffer, pH 6.8. Electrocatalytic reduction of H_2O_2 was monitored quantitatively using the peak at −400 mV (see Section 3.3) by both square wave and cyclic voltammetry.

Square wave responses for electrocatalytic reduction of H_2O_2 at this Pt/PANI/HRP biosensor can be observed in Fig. 4. When 1 mM H_2O_2 was added to the phosphate buffer, pH 6.8, under diffusion-controlled conditions, an initial increase in the current of the peak at −400 mV occurred, demonstrating effective electrocatalytic reduction of H_2O_2. Subsequent additions of H_2O_2 to the buffer solution resulted in both a positive shift in the reduction peak of about +190 mV and an increase in the magnitude of this reduction peak, proportional to H_2O_2 concentration.

Cyclic voltammetric responses for electrocatalytic reduction of H_2O_2 at this Pt/PANI/HRP biosensor can be observed in Fig. 5. When 1 mM H_2O_2 was added to the phosphate buffer (pH 6.8) under diffusion-controlled conditions, similar behavior to the square wave results was seen. The reduction peak current shifted and increased with increasing concentration of H_2O_2. These measurements were carried out at 5 mV s^{-1}. Comparison of the voltammograms with and without H_2O_2 illustrated that the PANI can effectively mediate electron transfer between peroxidase and a platinum electrode surface. Due to a high background current, lower hydrogen peroxide concentrations could not be studied. The minimum concentration that could be studied with this biosensor was 2.5×10^{-4} M.

For both voltammetric methods, linear relationships between catalytic current and H_2O_2 concentration within the range studied (2.5×10^{-4} to 5×10^{-3} M) were observed. Cyclic voltammetry data was used for further kinetic analysis. This linear correlation corresponds to the enzyme kinetic case where the substrate concentration is lower than the apparent Michaelis–Menten constant, K'_M, where the Michaelis–Menten kinetics are given as:

$$I = \frac{I_{max}[H_2O_2]}{[H_2O_2] + K'_M} \quad (2)$$

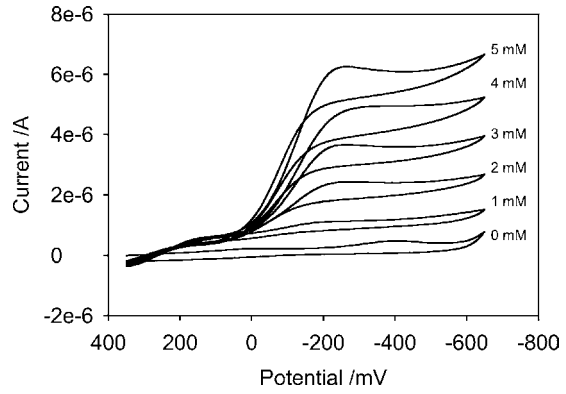

Fig. 5. Cyclic voltammograms of the Pt/PANI/HRP electrode in the presence of different concentrations of H_2O_2. Experimental conditions were: 0.1 M phosphate buffer (pH 6.8) and 5 mV s^{-1} scan rate.

where I is the observed catalytic current, I_{max} is the maximum obtainable current for the biosensor, and $[H_2O_2]$ is the bulk solution concentration of hydrogen peroxide. This can then be simplified to

$$I = \frac{I_{max}}{K'_M}[H_2O_2]. \quad (3)$$

The slope of the above plot (I_{max}/K'_M) represents the sensitivity of the biosensor and was found to be 2.88×10^{-2} A mol^{-1} dm^3. The reason for such a low sensitivity could be attributed to a low protein concentration used in the immobilization process (0.08 g l^{-1} for the present study), the low D_e obtained (as compared to Iwuoha et al.) and the low purity of the enzyme used (type II HRP). Similar biosensor formats have been reported and it has been shown that optimum performance is achieved when much higher concentrations of HRP were used for immobilization, specifically between 0.6 and 0.75 g l^{-1} [21,22]. Below this concentration level, it was found that surface coverage was not maximized leading to low signals, while at concentrations higher than 0.75 g l^{-1}, the response of the biosensor diminished, attributed to steric hinderence and hence, impeded electron transfer. However, up to a concentration level of about 0.6 g l^{-1}, increases in sensitivity were consistently reported

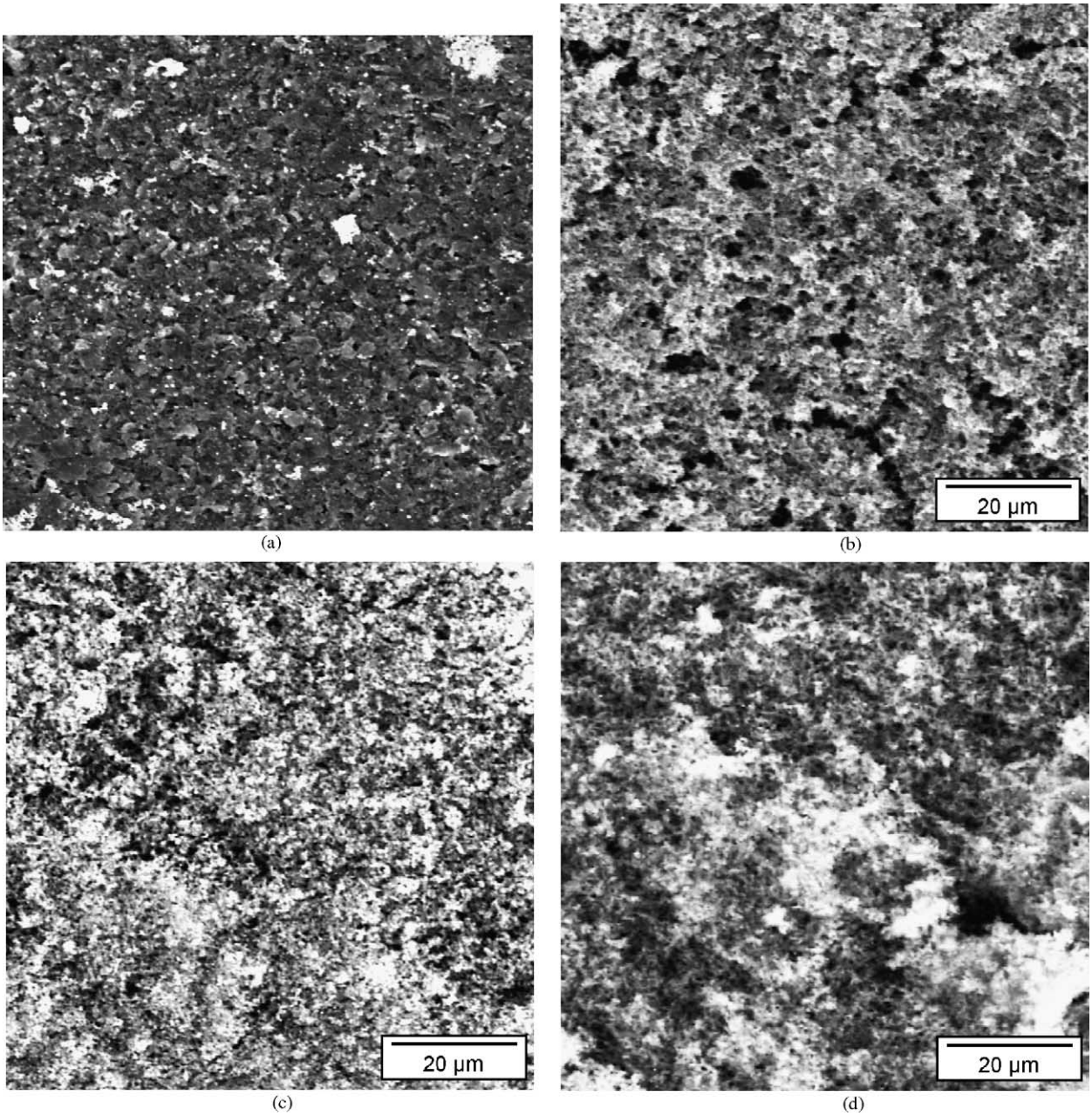

Fig. 6. SEM images of screen-printed electrodes (area, 0.09 cm^2): (a) bare SPE, (b) PANI-modified, (c) PANI/HRP-modified, and (d) PANI/HRP (in the presence of 1 mM)-modified SPEs, respectively. The accelerating voltage and magnification for all the images were 15 kV and 1500×, respectively.

[23]. Therefore, by increasing the protein loading on the surface of the PANI layer, the sensitivity of this biosensor may be enhanced. The linear range was between 2.5×10^{-4} and 5×10^{-3} M and r^2 was 0.995.

3.5. Scanning electron microscopy

SEM studies were carried out on screen-printed carbon electrodes. Fig. 6a shows the surface of a typical SPCE. The surface topography showed good definition of graphite particles. Fig. 6b shows that the growth of PANI from acidic media resulted in a sponge-like, branched, porous-structured, high-surface area polymer film on a SPCE, ideal for inclusion of enzyme. The enzyme immobilization ($0.08\ g\ l^{-1}$) can be observed by a change in topology to a speckled, grainy image (Fig. 6c), each of the speckles representing clusters of protein on the surface of the polymer. Following the performance of a cyclic voltammetry in the presence of H_2O_2 (1 mM), the morphology of the enzyme layer underwent a change (Fig. 6d). The many clusters of protein observed in Fig. 6c lessened, and the polymer layer beneath the protein could be seen clearly again. This deterioration in enzyme coverage may be due to the fact that a weak electrostatic immobilization method was employed and leaching of the enzyme into bulk solution upon application of potential. Another reason may be due to HRP inhibition by high concentration of H_2O_2. Further SEM and stability studies must be employed to elucidate the reasons for these results.

4. Conclusion

Electrochemical characterization of the electroactive polymer, polyaniline, was carried out on the surface of a platinum disk electrode. This electroactive polymer was then successfully applied in a horseradish peroxidase-based biosensor. It served as an efficient non-diffusional mediator, shuttling electrons between the redox active center of the enzyme and the electrode surface and also acted as a point of attachment for the enzyme. This Pt/PANI/HRP electrode effectively catalyzed the reduction of hydrogen peroxide, and both square wave and cyclic voltammetry were used to monitor the catalytic current. The linear range of this biosensor was from 2.5×10^{-4} to 5×10^{-3} M and r^2 was estimated at 0.995. Optimizing the protein loading on the surface of the Pt/PANI electrode could potentially increase the sensitivity of this biosensor. Spectroelectrochemistry (results not presented) and SEM studies provided an interesting insight into the behavior of the biosensor.

Although the analytical performance of this biosensor is not as good as reported in the literature, this biosensor has several advantages such as control of the polymer film characteristics, good signal amplification, ease of fabrication, and application to screen-printed electrodes for disposable use.

Acknowledgements

Financial support of National Research Foundation, South Africa is gratefully acknowledged. One of the authors, Aoife Morrin, would like to thank the Royal Society of Chemistry and Enterprise Ireland for financial support. The authors are grateful to Mr. Basil Julies and Mr. Nolan Botha of the Department of Physics, University of the Western Cape for assistance with SEM measurements.

References

[1] M. Gerard, A. Chaubey, B.D. Malhotra, Biosens. Bioelectron. 17 (2002) 345.
[2] S. Mu, C. Chen, J. Wang, Synth. Met. 88 (1997) 249.
[3] M. Kraljić, Z. Mandić, Lj. Duić, Corr. Sci. 45 (2003) 181.
[4] M.C. Bernard, A. Hugot-Le Goff, S. Joiret, P.V. Phong, Synth. Met. 119 (2001) 283.
[5] A. Mirmohseni, R. Solhjo, Eur. Polym. J. 39 (2003) 219.
[6] M. Malta, E.R. Gonzalez, R.M. Torresi, Polymer 43 (2002) 5895.
[7] S. Mu, H. Xue, Sens. Actuators B 31 (1996) 155.
[8] E.I. Iwuoha, D. de Villaverde, N. Garcia, M.R. Smyth, J. Pingarron, Biosens. Bioelectron. 12 (1997) 749.
[9] A. Killard, Presented at INABIS 1998—5th Internet World Congress on Biomedical Sciences at McMaster University, Canada, 7–16 December.
[10] A.N. Ivanov, L.V. Lukachova, G.A. Evtugyn, E.E. Karyakina, S.G. Kiseleva, H.C. Budnikov, A.V. Orlov, G.P. Karpacheva, A.A. Karyakin, Bioelectrochemistry 55 (2002) 75.
[11] E.M. Genies, C. Tsintavis, J. Electroanal. Chem. 195 (1985) 109.
[12] W. Schuhmann, C. Kranz, H. Wohlschläger, J. Strohmeier, Biosens. Bioelectron. 12 (1997) 1157.
[13] Y. Yang, S. Mu, J. Electroanal. Chem. 432 (1997) 71.
[14] J.C. Cooper, E.A.H. Hall, Biosens. Bioelectron. 7 (1992) 473.
[15] K.R. Prasad, N. Munichandraiah, Synth. Met. 123 (2001) 459.
[16] E.I. Iwuoha, M.R. Smyth, Polymer-based amperometric biosensors, in: M.E.G. Lyons (Ed.), Electroactive Polymer Electrochemistry, Plenum Press, New York, 1996, pp. 297–325.
[17] S.S. Razola, B.L. Ruiz, N.M. Diez, H.B. Mark Jr., J.-M. Kauffmann, Biosens. Bioelectron. 17 (2002) 921.
[18] A. Morrin, A.J. Killard, M.R. Smyth, Anal. Lett. 36 (2003) 2021.
[19] A.J. Bard, L.R. Faulkner, Electrochemical Methods: Fundamentals and Applications, second ed., Wiley, New York, 2000.
[20] L. Mao, K. Yamamoto, Talanta 51 (2000) 187.
[21] A. Morrin, A. Guzman, A.J. Killard, J.M. Pingarron, M.R. Smyth, Biosens. Bioelectron. 18 (2003) 715.
[22] A.J. Killard, L. Micheli, K. Grennan, M. Franek, V. Kolar, D. Moscone, I. Palchetti, M.R. Smyth, Anal. Chim. Acta 427 (2001) 173.
[23] T. Tatsuma, M. Gondaira, T. Watanabe, Anal. Chem. 64 (1992) 1183.