Amperometric biosensor based on a nanoporous ZrO_2 matrix

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Abstract

A biosensor was investigated based on the use of ZrO_2 sol–gel matrix for enzyme immobilization in the mild condition. This bioceramic zirconia alcogel has been prepared by the novel alcohothermal route with a cheap inorganic salt $Zr(NO_3)_4 \cdot 5H_2O$ with several desirable features including a large surface area (about $460\,m^2\,g^{-1}$) as well as pore volume and a well-developed textural mesoporosity, and horseradish peroxidase was selected as a model enzyme. The results of transmission electron microscopy (TEM) and BET measurement of the substrate showed that the as-prepared zirconia matrix has an advantageous microenvironment and large surface area available for high enzyme loading. The parameters affecting both the entrapment of enzyme and the biosensor response were optimized. The resulting biosensor exhibited high sensitivity of $111\,\mu A\,mM^{-1}$ for hydrogen peroxide over a wide range of concentrations from 2.5×10^{-7} to $1.5 \times 10^{-4}\,mol\,l^{-1}$, quick response of less than 10 s and good stability over 3 months.
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1. Introduction

The development of new materials that can improve the analytical capacities of sensor devices is an important aspect of biosensor technology. Porous inorganic sol–gel materials have opened up a new route for the fabrication of chemical sensors and biosensors due to the attractive properties of sol–gel materials, including the simplicity of preparation, tunable porosity, low temperature encapsulation, negligible swelling, mechanical and biodegradational stability [1–12]. Reviews were presented of the state-of-the art of electrochemical biosensors employing sol–gel materials [4–6,13,14]. Since Braun et al. [1] demonstrated the possibility of protein immobilization in sol–gel silica matrices. This class of ceramic materials has been further applied to produce silica-based analysis detector. However, the recent literature seems to show that much attention has been paid to use of conductive bioceramic metal oxide materials. The physical and chemical characteristics of metal oxides can be easily manipulated, giving easy control over polarity, pore size distribution, and electronic properties. Glezer and Lev took advantage of the high conducting of vanadium pentaoxide sol–gel for designing a glucose biosensor [15]. Topoglidis et al. [16,17] reported the immobilization and bioelectrochemistry of proteins on nanoporous TiO_2 and ZnO films, respectively. Cosnier et al. [18] investigated a glucose biosensor within polypyrrole films electrodeposited on TiO_2. Wang and Pamidi [19] reported the new gold-ceramic electrodes for electroanalytical applications by adding the enzyme to the sol–gel solution by dispersion of the metal power. Many new amperometric biosensors based on novel sol–gel

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composite have offered great prospects for analytical applications.

In addition, considering the protein adsorption on nanocrystalline films was mainly electrostatic and controlled by the pH, the protein charge and the solution ionic strength [16,17]. Here, we extended the study to an attractive matrix, ZrO_2 by the sol–gel method. Its isoelectric point is about 4.15 [20], which should be more suitable for the adsorption of high IEP protein.

Zirconia is a technological important material that has recently attracted considerable interest in practical applications [21]. It is a unique material of excellent chemical inertness and biocompatibility feature; moreover its surface has both oxidizing and reducing properties, as well as acidic and basic properties [22]. It has been reported that titania- and zirconia-sol–gel carbon composite electrodes were used as substrates for decrease in the overvoltage of NADH oxidation reaction [23]. Dong and Kuwana's research [24] showed that the dispersion of numerous metal oxides such as SiO_2, ZrO_2 on electrode surfaces enhanced the oxidation rate of hydroquinone, which attributed to the adsorption of the electroactive species and the acceleration of the proton transfer step. Therefore, in electrochemical bioanalysis, it should be studied as an important promising candidate for catalysts and support material. However, the use of highly expensive starting material was required, which strongly limited the possible fabrication of a biosensor. We are not aware of any previous reports of such more convenient and economical methodology to prepare ZrO_2 matrix for protein immobilization. Here, the preparation, characterization and electroanalytical utility of a stable zirconia sol–gel-derived biosensors have been described.

In this paper, we presented for the first time a novel enzyme biosensor based on a mesoporous ZrO_2 matrix readily prepared by sol–gel procedure using a cheap inorganic salt $[Zr(NO_3)_4 \cdot 5H_2O]$ as starting material in the neutral condition. The results showed that this novel synthesis route affords the preparation of the zirconia with several desirable features including a large surface area as well as pore volume and a well-developed porosity in the mesoporous range, providing a new economical approach for protein immobilization. It suggested that the use of ZrO_2 sol–gel might be advantageous for the development of biosensors. The resulting biosensor exhibited high sensitivity

of $111 \mu A\,mM^{-1}$, quick response and good stability. A comparison of the PGE/PTH/HRP zirconia sol–gel based biosensor with the PGE/ZrO_2 based sensor was also presented.

2. Experimental

2.1. Reagents

Horseradish peroxidase was purchased from Sigma (HRP, E.C.1.11.1.7, $A > 250$ units/mg), thionine (TH) from Fluka, $Zr(NO_3)_4 \cdot 5H_2O$ and H_2O_2 (30% (w/v)) solution was purchased from Shanghai Chemical Reagent Company. All other chemicals were of analytical grade and were used without further purification. All solutions were prepared with the second-distilled deionized water.

2.2. Apparatus

A three-electrode system was employed, with a platinum foil counter electrode, a saturated calomel reference electrode (SCE), and the biosensor working electrode. Amperometric measurements were carried out on CHI 660A (CH Instrument Electrochemical Workstation, USA). A magnetic stirrer and bar with a constant-temperature control provide the convective transport.

Transmission electron microscopy (TEM) images of ZrO_2 sol–gel matrix was taken with a Hitachi H-800, using an accelerating voltage of 200 kV (Hitachi, Tokyo, Japan). Fourier transform infrared (FT-IR) spectra measured by KBr pellets containing the sol–gel membranes or tyrosinase/sol–gel membranes, were obtained in the range of 2000–500 cm^{-1} on an Avatar 360 spectrometer (Nicolet, USA) at room temperature. The surface area of the samples were measured using the BET method by N_2 adsorption and desorption at 77 K in a micrometrics ASAP2000 system.

2.3. Preparation of zirconia sol–gel solution

According to our preparation [25], appropriate amount $Zr(NO_3)_4 \cdot 5H_2O$ were dissolved in 50 ml absolute ethanol to obtain a 0.3 M solution followed by putting the solution into an autoclave of 50 ml capacity. After sealed, the autoclave was maintained at

383 K for 60 min, and then allowed to cool to room temperature. A translucent alcogel was thus obtained. The ethanol gel was aged at room temperature for 180 min. A stable and homogeneous ZrO_2 sol–gel has been obtained (0.4 M) and used as a stock standard solution. This formulation could be changed when certain experimental parameters were investigated.

2.4. Construction of the H_2O_2 biosensor

A 4 mm pyrolytic graphite electrode (PGE) was used as the base electrode for the sol–gel-modified H_2O_2 biosensor. The PGE was polished by 0.3, 0.1, 0.05 μm alumina particles, rinsed thoroughly with deionied acetone and double-distilled water in an ultrasonic bath, and dried in air before use.

Firstly, the electropolymerization of thionine was carried out in two steps following our previous research. Holding the PGE under a constant potential of 1.5 V for 5 min and voltammetric cycles between -0.45 V and 0.05 V at $100\,mV\,s^{-1}$ in pH 6.5 phosphate buffer solution containing 0.1 mM thionine. All solutions tested were thoroughly deoxygenated by bubbling pure nitrogen, and a continuous flow of nitrogen was maintained over the solution during experiments unless stated otherwise.

Then the immobilization of HRP in the ZrO_2 sol–gel matrix was accomplished by addition of 10 μl of HRP ($10\,mg\,ml^{-1}$) completely with 10 μl ZrO_2 sol–gel. With a micropipet, 10 μl aliquots of such a colloid were deposited on the PGE surface and drying under 4 °C in air. Then the biosensor was prepared (PGE/PTH/HRP-sol–gel). The sensor was kept in air at 4 °C between measurements. As the comparison, PGE/ZrO_2 electrode without enzyme was also prepared.

2.5. Measurement procedure

Cyclic voltammetric and amperometric measurements were performed with CHI 660 electrochemical workstation (CHI, Cordiva, USA). The three-electrode system was used for detection. All the experimental solutions were thoroughly deoxygenated by bubbling nitrogen through the solution all over the experiment.

In the constant potential experiments, successive additions of stock H_2O_2 solution to the phosphate buffer were made and the current–time data were recorded as a function of time following the addition of H_2O_2.

3. Results and discussion

3.1. Interaction between ZrO_2 sol–gel and HRP

ZrO_2 sol–gel has a porous structure of matrix for embedding the enzymes. To investigate the microstructures of the as-synthesized ZrO_2 matrix and the immobilization of enzyme in the matrix, transmission electron microscopy images were experimented. From Fig. 1, it can be seen that the matrix was highly porous in nature, which consisted of clusters of cross-linked particles about 5 nm. The TEM picture of ZrO_2 matrix also displayed a three-dimensional uniform porous structure, which provided a significant increase of effective electrode surface for enzyme loading and good preparation reproducibility. The surface area and the pore volume of the matrix, measured using the BET method by N_2 adsorption and desorption, were about $460\,m^2\,g^{-1}$ and $2.9\,cm^3\,g^{-1}$, respectively.

The interactions between ZrO_2 matrix and HRP were studied by infrared spectra. The matrix has adsorption bands at 781 and $1054\,cm^{-1}$, characteristics of frame asymmetric and symmetric flexible vibrations,
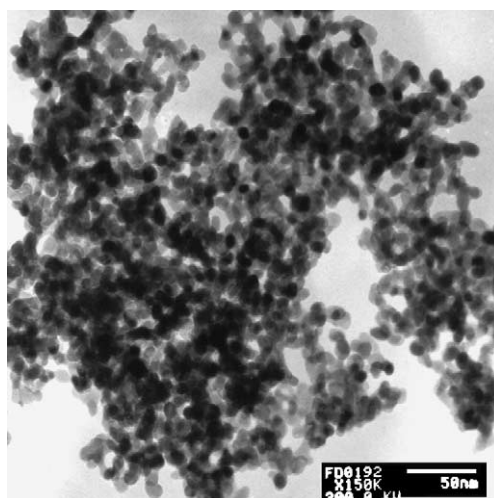

Fig. 1. The transmission electron microscopy image of the as-synthesized ZrO_2 matrix and the immobilization of enzyme in the matrix.

and HRP has specific adsorption bands at 729 and $1651 \, cm^{-1}$. When both of them mixed, the specific adsorption bands of HRP still exist, while some of bands of matrix nearly disappeared and the band at $1640 \, cm^{-1}$ shifted in the IR spectrum of ZrO_2-HRP. These results sowed that there might be intermolecular interaction between enzyme and some specific sites of matrix, and apparently the ZrO_2 sol–gel was a good immobilization matrix for enzyme loading.

3.2. Fabrication of the biosensor

As in our previous paper [26] about the electropolymerization of thionine on glassy carbon electrode, the pyrolytic graphite electrode was preanodized at a constant potential of 1.5 V for 5 min, then was electropolymerized in pH 6.5 phosphate buffer solution containing 0.1 mM thionine from 0.05 V to −0.45 V. The cyclic voltammogram (Fig. 2) indicated the growth process of the PTH films with consecutive cycles. When the PTH/PGE electrode was washed thoroughly and then removed to blank phosphate buffer solution without thionine, the current existed, which confirmed that the polymer film has formed on the PGE surface.

The cyclic voltammogrames of the thionine modified electrode at various scan rates were studied. The peak currents were linearly proportional to the scan rates, which indicated a thin film adsorption redox behavior. In addition, the experiment results showed that electrochemical behavior of the modified electrode was influenced by pH, and the $E^{0\prime}$ value decreased by 55 mV/pH between pH 6 and 8.5 for the polymerized thionine, which was closed to the Nernstain value of 59 mV for a two-electron, two-proton process.

Additionally, the film thickness, pH and the HRP/ ZrO_2 concentration are the important factors to influence the properties of the matrix structure and the performance of the biosensor. The thickness of the sol–gel film and the response of the immobilized enzyme were affected by varying the ratio of ZrO_2 stock standard sol–gel to water and ZrO_2/HRP. The results in Fig. 3 showed that the sensitivity and dynamic response range of the biosensor increased when the ratio of ZrO_2/H$_2$O was smaller than 1:2. If the ratio was lower than 1:8, the response of the biosensor gradually decreased. Thus, the optimum ratio was fixed at 1:8 for the immobilizing HRP, and the surface of the sol–gel-modified enzyme electrode appeared to be smooth and firm under such condition. Then the effect of the HRP $(10 \, mg \, ml^{-1})$/ZrO_2 ratio on the biosensor response was further studied and the results showed that the optimum ratio was fixed at 1:1 for the preparation of the biosensor.

The pH of the sol–gel fabrication process might influence the performance of the biosensor. The zero point charge values of the supports are of importance in the immobilization of enzymes since at pH values above the ZPC the surface of the matrix would be negatively charged, and conversely, at pH values
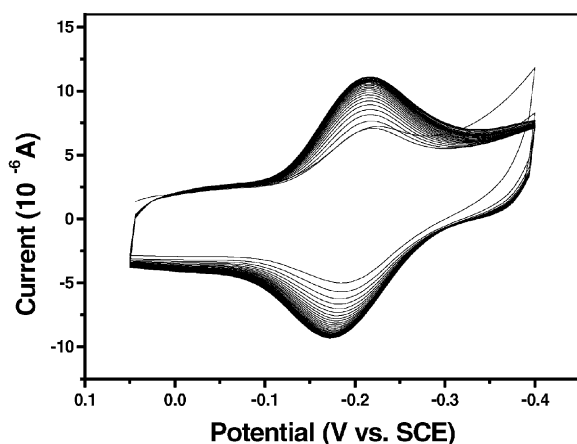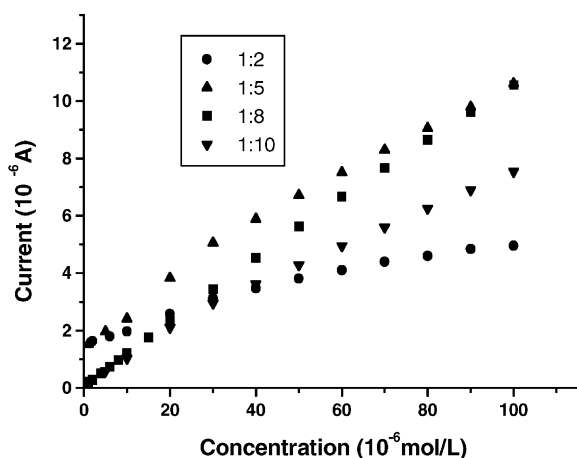

Fig. 2. The cyclic voltammograms of PTH progressive course on a preanodized the pyrolytic graphite electrode at 1.5 V for 5 min in pH 7.0 buffer containing 0.1 mM TH.

Fig. 3. Optimization of the concentration of ZrO_2 sol–gel (0.4 M ZrO_2:HO$_2$) on the response of the biosensor.

below the ZPC the surface would be positively charged. It has been reported that the isoelectronic point of Horseradish peroxidase is 8.9, while ZrO_2 has an IEP measurement of 4.18 [20]. Our experiments showed that the optimum response of the biosensor was obtained at about pH 6–7 for immobilizing HRP, which was in good agreement with the optimum activity of HRP.

3.3. Amperometric response of the biosensor

Fig. 4 showed the cyclic voltammograms of the H_2O_2 biosensor in a deoxygenated phosphate buffer solution without H_2O_2 (a) and with H_2O_2 (b). In the absence of H_2O_2, the biosensor gave no response and only the typical electrochemical behavior of the electropolymerized thionine was observed. Addition of H_2O_2 in buffer solution, the reduction current increased with no increase in the oxidation current, which indicated that a catalytic reaction occurred on the biosensor. This result showed that thionine could effectively shuttle electrons between the redox center of HRP and the electrode. The process of electron transfer between H_2O_2 and the biosensor was illustrated in the following paragraph.

At first, HRP reduced H_2O_2 diffusing from solution into the membrane and oxidizes HRP to HRP-I

$$H_2O_2 + HRP \rightarrow HRP\text{-}I + H_2O$$
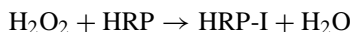
The reduction of HRP-I to HRP includes two separate steps

$$HRP\text{-}I + PTHH \rightarrow HRP\text{-}II + PTH^\bullet$$

$$HRP\text{-}II + PTH^\bullet \rightarrow HRP + PTH^+$$
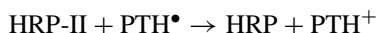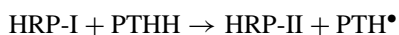
where HRP-I and HRP-II are two unstable intermediates. PTHH and PTH^+ represent reduced and oxidized forms of poly(phenazine methosulfate). Subsequently oxidized PTH^+ can be recycled to be reduced to PTHH at the electrode, bring about a reduction current

$$PTH^+ + H^+ + 2e \rightarrow PTHH$$
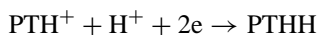
The pH dependence of the electrocatalytic current for the biosensor decided in two aspects: one was the pH effect of the activity of HRP; the other was that of the peak potentials of thionine. Considering these two aspects, the effect of pH on the biosensor was tested and an optimum pH 6.8 was selected in this work (Fig. 5), which was in agreement with that soluble HRP reported before [27]. Therefore the ZrO_2 matrix did not change the optimal pH value for the bioelectrocatalytic reaction of the immobilized HRP to H_2O_2.

The potential dependence of the amperometric signal and background current of the biosensor was
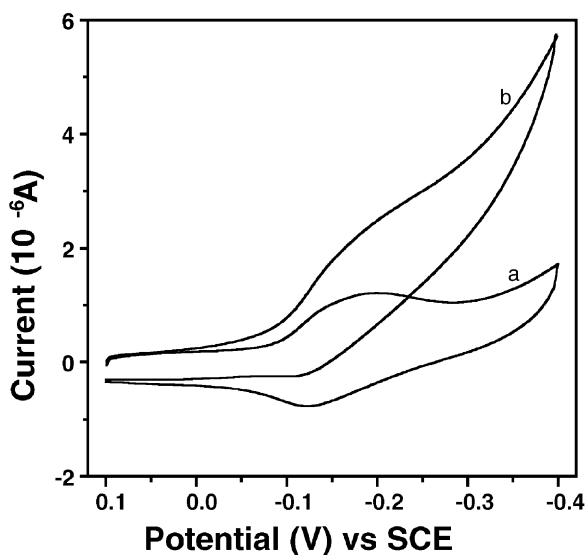

Fig. 4. Cyclic voltammograms of the biosensor in: (a) the absence; and (b) presence of H_2O_2 at a scan rate of $100\,mV/s$.
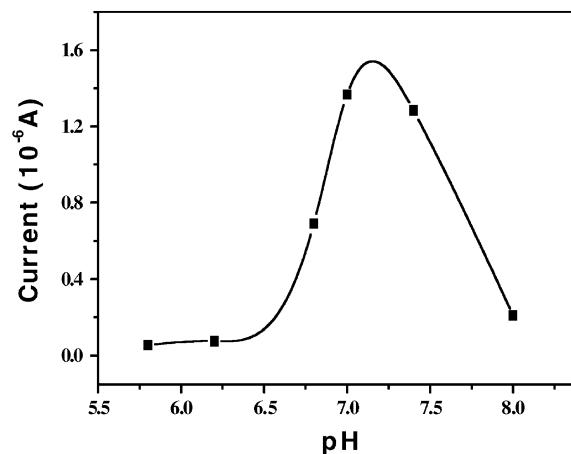

Fig. 5. Effect of pH on the H_2O_2 biosensor at $25\,°C$.
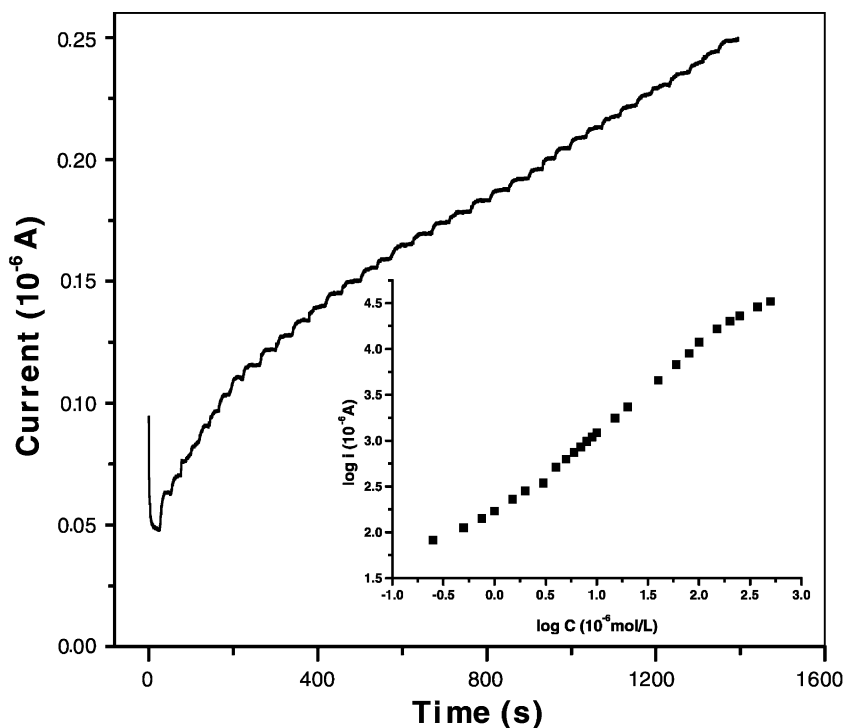
Fig. 6. A typical current–time response curve for successive addition of 25 μl, 1 mmol l⁻¹ H₂O₂ to 10 ml buffer solution kept under stirring for an enzyme electrode fabricated under optimized condition. Operation potential −0.2 V vs. SCE; insert curve is a calibration of the biosensor.

tested. The results showed that the highest ration of signal-to-background was achieved at -0.2 V. The low operational potential minimized interferences from other coexisting electroactive species.

Fig. 6 displayed a typical amperometric response of the biosensor after the addition of successive aliquots of H_2O_2. A defined reduction current proportional to the H_2O_2 concentration was observed. The time was less than 10 s to reach 95% of the steady-state value, which was attributed to the fast diffusion of the substrate in the porous film. The biosensor exhibited a linear calibration range from 2.5×10^{-7} to 1.5×10^{-4} mol l⁻¹ with a sensitivity of 111 μA mM⁻¹ and a regression equation: $I(\mu A) = 0.081 + 0.111 C(\mu M)$ (in Fig. 7) and $\log I(\mu A) = 2.24 + 0.87 \log C(\mu M)$ (in Fig. 6 insert curve), respectively. The detection limit was 1.0×10^{-7} mol l⁻¹ at a signal-to-noise ratio of 3. Such a high sensitivity may be attributed to the favorable microenvironment of the ZrO_2 matrix, which

has a general affinity for the protein loading and could stabilize its biological activity to a large extent.

In order to demonstrate the catalytic function of ZrO_2 to H_2O_2, the amperometric characterization of the ZrO_2-HRP/PGE and ZrO_2/PGE were compared. From Fig. 7 and insert curve, the sensitivity of the former was about 10 times greater than that of the latter. The ZrO_2/PGE electrode exhibited a linear range from 2.0×10^{-4} to 3.5×10^{-2} mol l⁻¹ with a detection limit of 1.0×10^{-6} mol l⁻¹.

The developed biosensor based on the ZrO_2 matrix displayed good reproducibility. The relative standard deviation determined by 20 successive assays of a 5.0×10^{-5} mol l⁻¹ H_2O_2 sample was 2.7%. As to a series of six biosensors, which made independently under the same conditions, a relative standard deviation of 7.5% was obtained for the individual current responses to 5.0×10^{-5} mol l⁻¹. In addition, the accuracy of the sensor was evaluated by determining
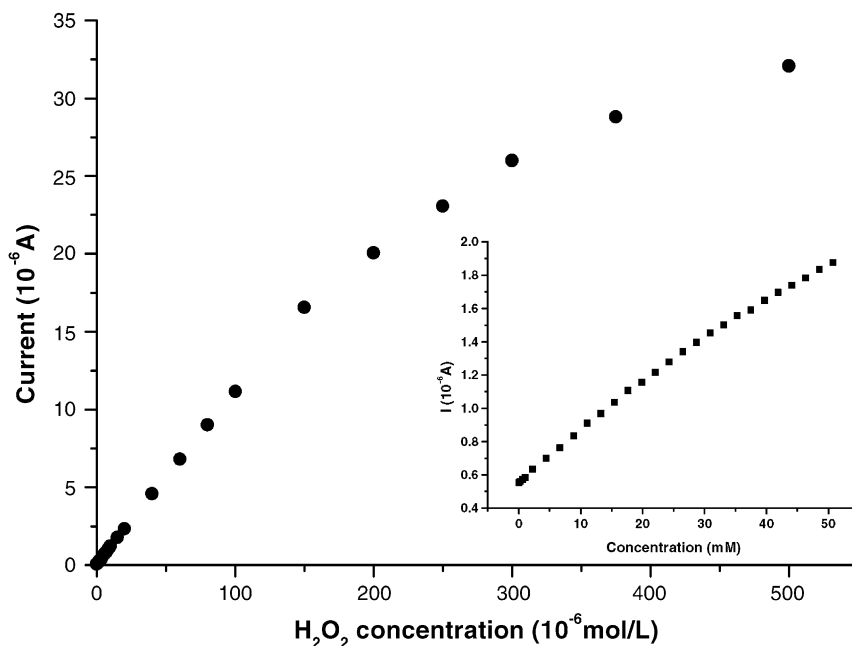
Fig. 7. Calibration curves of the ZrO$_2$-HRP/PGE and ZrO$_2$/PGE electrode (insert curve).

the recoveries of hydrogen peroxide after standard addition of concentrations ranging from 8.5×10^{-6} to 7.5×10^{-4} M in sterilized milk samples. The biosensor showed satisfactory results with an average recovery of 100.3%.

The operational stability was investigated by consecutive measurements of a 7.5×10^{-5} mol l^{-1} H$_2$O$_2$ sample. After 160 measurements in 5 h, the biosensor retained about 82% of its initial activity, and no swell of the HRP/ZrO$_2$ sol–gel membrane was observed in the solution during this continuous measurements. Additionally, the long-term stability of the ZrO$_2$ sol–gel-derived biosensor was studied. The response of the biosensor was tested day after day during the first 2 weeks, afterwards the experiments were periodically carried out over a period of 3 months and dry storage at 4 °C. The results showed that the response decreased about 4.5% of its initial sensitivity after 2 weeks and remained 75% after 3-month usage, while the dynamic linear range of the biosensor decreased gradually with the storage time. This long-term stability was attributed to the interaction between HRP and the matrix. The amine and carboxyl groups of the enzyme might interact with some

specific sites of the ZrO$_2$ substrate, which prevent the enzyme from leaking out of the ZrO$_2$ sol–gel membranes.

4. Conclusion

The biosensor based on immobilization of HRP in a ZrO$_2$ sol–gel matrix on an electropolymerized thionine modified electrode offered advantage characteristics, including high sensitivity and long-term stability. A novel and easy alcohothermal process has been developed to prepare the stable zirconia sol–gel using a cheap inorganic salt as the starting material with several desirable characteristics including a large surface area and a well-developed porosity in the mesoporous range, providing a favorable microenvironment for enzyme binding. The results showed that the zirconia sol–gel was an attractive matrix for enzyme immobilization and electroanalytical measurements. No loss of enzyme was observed in the sol–gel membrane and enzyme activity was maintained. It could be extended to other proteins to fabricate the biosensors.

Acknowledgements

References

[1] S. Braun, S. Rappoport, R. Zusman, D. Avnir, M. Ottolenghi, Mater. Lett. 10 (1990) 1.

[2] D. Avnir, S. Braun, M. Ottolenghi, in T. Bein (Ed.), Proceedings of the ACS Symposium Series, No. 499 on Synthetic Control in Thin Films and Solids, American Chemical Society, Washington, DC, 1992, pp. 384–404.

[3] L.M. Ellerby, C.R. Nishida, F. Nishida, S.A. Yamannaka, B. Dunn, J.S. Valentine, J.I. Zink, Science 255 (1992) 1113.

[4] O. Lev, M. Tsionsky, L. Rabinovich, V. Glezer, S. Sampath, I. Pankratov, J. Gun, Anal. Chem. 67 (1995) 23A.

[5] O. Lev, Analysis 20 (1992) 543;
S. Sampath, O. Lev, Anal. Chem. 68 (1996) 2015.

[6] B.C. Dave, B. Dunn, J. Valentine, J.I. Zink, Anal. Chem. 66 (1994) 1120A.

[7] D. Avnir, S. Braun, O. Lev, M. Ottolenghi, Chem. Mater. 6 (1994) 1605.

[8] R.A. Dunbar, J.D. Jordan, F.V. Bright, Anal. Chem. 68 (1996) 604.

[9] J. Wang, P.V.A. Pamidi, D.S. Park, Anal. Chem. 68 (1996) 2705.

[10] U. Narang, P.N. Prasad, F. Bright, K. Ramanathan, N. Kumar, B. Malhorta, M. Kamalasana, S. Chandra, Anal. Chem. 66 (1994) 3139.

[11] P. Audebert, C. Demaille, C. Sanchez, Chem. Mater. 5 (1993) 911.

[12] Z. Liu, B. Liu, J. Kong, J. Deng, Anal. Chem. 72 (2000) 4707.

[13] J. Wang, Anal. Chim. Acta 399 (1999) 21.

[14] J. Lin, C.W. Brown, Trends Anal. Chem. 16 (1997) 200.

[15] V. Glezer, O. Lev, J. Am. Chem. Soc. 115 (1993) 2533.

[16] E. Topoglidis, A.E.G. Cass, G. Gilardi, S. Sadeghi, N. Beaumont, J.F. Durrant, Anal. Chem. 70 (1998) 5111.

[17] E. Topoglidis, A.E.G. Cass, B. O'Regan, J.R. Durrant, J. Electroanal. Chem. 517 (2001) 20.

[18] S. Cosnier, A. Senillou, M. Gratzel, P. Comte, N. Vlachopoulos, N. Renault, C. Martelet, J. Electroanal. Chem. 469 (1999) 176.

[19] J. Wang, P.V.A. Pamidi, Anal. Chem. 69 (1997) 4490.

[20] T. Klimova, M.L. Rojas, P. Castillo, R. Cuevas, J. Ramirez, Microporous Mesoporous Mater. 20 (1998) 293.

[21] D.T. On, D. Desplantier-Giscard, C. Danumah, S. Kaliaguine, Catal. Today 20 (1994) 199.

[22] M.A. Aramendia, V. Borau, C. Jimenez, J.M. Marinas, A. Marinas, A. Porras, F.J. Urbano, J. Catal. 183 (1999) 240.

[23] J. Wang, P.V.A. Pamidi, M. Jiang, Anal. Chim. Acta. 360 (1998) 171.

[24] S. Dong, T. Kuwana, J. Electrochem. Soc. 131 (1984) 813.

[25] J. Hu, Y. Cao, J. Deng, Chem. Lett. 5 (2001) 398.

[26] R. Yang, C. Ruan, W. Dai, J. Deng, J. Kong, J. Electroanal. Chem. 44 (1999) 1585.

[27] A.C. Maehly, Methods Enzymol. 11 (1955) 807.