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A novel hemin-based organic phase artificial enzyme electrode and its application in different hydrophobicity organic solvents

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ABSTRACT

Based on the newly discovered artificial enzyme formed by mixing hemin with supramolecular hydrogels via the self-assembly of amphiphilic oligopeptides, we prepared a novel organic phase artificial enzyme electrode by coating the artificial enzyme on an electrode which was then covered with sodium alginate for protection. Scanning electron micrograph showed that the supramolecular hydrogel kept its nanofibers structure on the electrode surface. Hemin dispersed in the supermolecular hydrogel as monomer greatly promotes its direct electrochemistry behavior in organic solvents. At the same time, this electrode exhibited higher electrocatalytic ability to tert-butyl hydroperoxide (TBHP) than free hemin modified electrode (free hemin mainly present as dimer). As low as 27 μM TBHP could be detected with a linear range from 6.6×10^{-5} to 1.27×10^{-2} M via amperometric method. The biosensor can reach 95% of the steady-state current in about 10 ± 2 s. More importantly, it can be applied in both hydrophilic and hydrophobic solvents without adding extra buffer or mediators to them that cannot be received by most traditional organic phase enzyme electrodes. This unique property greatly promotes the development of the organic phase enzyme electrodes by facilitating the detection of different kinds of substrates of the hemin-based artificial enzyme soluble in hydrophilic and hydrophobic solvents. The artificial enzyme electrode was successfully used to determine organic peroxides in body lotion samples.

1. Introduction

Biosensors and biocatalysis for nonaqueous systems are rarely reported compared to those in aqueous solutions due to the hostile environment of organic solvents for natural enzymes. The organic phase enzyme electrodes (OPEEs) usually bear some serious disadvantages, such as easily denaturalization, low activities and requiring "essential hydration layer" (Fatibello-Filho and Cruz Vieira, 2000) to maintain activity which greatly limit their applications in this field. Although OPEEs have advanced a lot due to the application of some advanced materials to protect natural enzymes (Guo and Dong, 1997; Wang and Dong, 2000; Yu and Ju, 2004), most of the OPEEs still have some disadvantages, such as easily losing the essential hydration layer in pure organic solvents, poor catalysis in hydrophilic organic solvents. Although a few studies have reported OPEEs in pure organic solvents modified with natural enzyme engulfed in polyhydroxyl cellulose (Dong and Guo, 1994) and sol–gel matrix (Yu and Ju, 2004), the most popular and efficient way to maintain the activity of the enzyme is to add extra water or buffer to the solvents (Morales et al., 2005; Wang and Dong, 2000; Campanella et al., 1999). Some researchers even made use of this "disadvantage" to develop the OPEEs for detecting water in food fats and cosmetic ointments (Wang and Julio Reviejo, 1993; Campanella et al., 2001a). Besides, it is hard to obtain the same catalytic current in hydrophilic solvents as in hydrophobic ones (Wu and Choi, 2003; Dong and Guo, 1994; Campanella et al., 1998). It is probably hydrophobic solvents are less likely to interact directly with the hydration layer around the enzyme molecule and therefore the enzyme can keep its activity (Fatibello-Filho and Cruz Vieira, 2000; Schubert et al., 1991; Zaks and Klibanov, 1988). Tetrahydrofuran (THF) and the hydrophilic solvents of *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) are excellent solvents for many analytes. But in previous studies, low or no catalytic current was obtained in these solvents (Dong and Guo, 1994; Fatibello-Filho and Cruz Vieira, 2000). Recently, catalysis for H_2O_2 in THF has been reported, but the signal is too noisy and irreproducible to be utilized in detection (Konash and Magner, 2006). Thus, it is necessary to further develop the OPEEs to meet the increasing demand for it.

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Currently, artificial enzymes have attracted intensive attentions due to their extraordinary properties and convenient applications in organic solvents (Li et al., 1998; Wang et al., 2007b, 2008). Artificial enzymes cover a variety of complexes based on different synthesis strategies, like the porphyrin-based mimetic enzymes (Crestini et al., 2004), cyclodextrin mimetic enzymes (Tabushi and Kuroda, 1984; Zhang and Breslow, 1997), molecularly imprinted polymers based mimetics (Toorisaka et al., 2003), etc. Recently, Fe_3O_4 nanoparticles were discovered as peroxidase mimic (Gao et al., 2007) which showed great catalysis ability to H_2O_2 (Wei and Wang, 2008). Among these artificial enzymes, a major part concerns the matrices which are used to mimic the peptidic group of natural enzymes to better facilitate the function of active sites in natural enzymes (Liu et al., 1999; Darbre and Reymond, 2006; Wang et al., 2007b, 2008). It was reported that microenvironment surrounding the active site of enzymes greatly influence the efficiency of the synthetic porphyrin (Suslick et al., 1985; Cook et al., 1986). Consequently, intensive developments were achieved by using different matrices to mimic the microenvironment of the natural enzyme. Nanogels (Wulff et al., 2006), dendrimers (Darbre and Reymond, 2006), cyclodextrins (Liu et al., 1999) and polymer hydrogel (Pizarro et al., 1997) were synthesized, discovered and applied as matrices for artificial enzymes in many fields. Sensors made from metalloporphine-based artificial enzymes have been reported early in 1985 (Saito et al., 1985). Subsequently, Ho and Rechnitz (1987) reported an electrochemistry biosensor where an artifcial enzyme was applied on a potentiometric gas-sensing membrane electrode (Ho and Rechnitz, 1987). Later, Fe–porphine complexes and β-cyclodextrin–hemin were used as peroxidase substitutes and achieved agreeable results in detection of H_2O_2 (Genfa and Dasgupta, 1992; Liu et al., 1999). In recent years, Baldini et al. (2002) successfully prepared porphyrin biomimetic modified electrodes for the detection of peroxides. However, all the mentioned mimetic enzymes were applied in aqueous solutions or mixture of organic solvents and buffer and the sensitivities to organic peroxides were lower than that of H_2O_2. For example, although hematin showed great catalytic activity to H_2O_2, but for methyl hydroperoxide, one of the organic peroxide, it exhibited only about 10% of the sensitivity displayed by horseradish peroxidase (HRP) (Genfa and Dasgupta, 1992). No reference related to the organic phase mimetic enzyme electrodes is reported, to our knowledge.

Recently, a kind of supramolecular hydrogels has drawn our attentions because of its superactivity exhibited by the enzyme immobilized in these gels (Wang et al., 2007a). Based on the supramolecular hydrogels, a new mimic of the peroxidase was synthesized by incorporating hemin in the supramolecular hydrogels and the catalytic activity was examined by testing its catalytic oxidation to pyrogallol and H_2O_2 in organic media (Wang et al., 2007b). The supramolecular-hydrogel-based artificial enzyme showed outstanding catalytic performance compared with that of other carriers (β-CD and polyacrylamide hydrogel). In this work, we take advantage of the newly discovered artificial enzyme and successfully used this material to prepare a novel electrochemical biosensor for the detection of tert-butyl hydroperoxide (TBHP). In Wang's report, the supramolecular-hydrogels skeleton was of great importance in reducing μ-oxo bihemin resulting from the dimerization of free hemin in water and the artificial enzyme showed better catalytic activity than free hemin which was also observed in our experiments. This new biosensor is different from traditional OPEEs due to its excellent catalytic response both in hydrophobic and hydrophilic solvents. More importantly, compared with lots of HRP modified electrodes, the artificial enzyme modified electrode exhibits better performance for TBHP in organic solvents because of its wider linear range and faster response time.

2. Experimental section

2.1. Reagents

Hemin, Fmoc-L-lysine (FLlys) and Fmoc-L-phenylalanine (FLphe) were purchased from Fluka. Histidine was supplied by Sigma. Tetrabutyl ammonium perchlorate (TBAP) as electrolyte in organic solvents was from TCI-GR. tert-Butyl hydroperoxide (65 wt%) was from Shanghai Chemical Reagents Co. (Shanghai, China). Sodium alginate powder was purchased from Jiangsu Huakang Technical Co. (Nanjing, China). The samples analyzed were a commercial body lotion (P&G) purchased in a local supermarket. HRP was from Sigma. All organic solvents were used as received and the water content is less than 0.1% (w/w) in THF, ethyl acetate, dichloromethane and DMF, 0.2% (w/w) in DMSO and 0.03% (w/w) in chloroform. All other chemicals were of analytical grade and used as received. Twice-distilled water was used throughout.

2.2. Apparatus

Amperometric and cyclic voltammetric (CV) experiments were performed on a CHI 660A electrochemical workstation (Shanghai Chenhua Apparatus, China). UV–vis spectroscopic experiments were carried out with a UV-2401P spectrophotometer (Shimadzu, Kyoto, Japan). Morphologies of the artificial enzyme modified glassy carbon slides were studied on an S-4800 II Ultra-high Resolution Scanning Electron Microscope (HITACHI, Japan). The samples were prepared by dropping 2.5 μl artificial enzymes on two glassy carbon slides which were allowed to dry in dryer container for half an hour and then covering one of them with 3.5 μl sodium alginate which was allowed to dry in room temperature. All experiments were carried out on a conventional three-electrode system with the modified glassy carbon electrode (GCE, 3 mm diameter) as the working electrode, the Ag/AgCl (saturated KCl) as the reference electrode and the Ag wire as the counter electrode.

2.3. Electrode preparation

The GC electrode was carefully polished with 1.0, 0.3 and 0.05 μm alumina slurry step by step and rinsed thoroughly with water between each step. At last, the electrode was sonicated in water and absolute ethanol successively and allowed to dry at room temperature. The mimetic enzyme was prepared according to the procedure previously described (Wang et al., 2007b) with a little modification. Adding sodium carbonate (100 mmol), Fmoc-L-phenylalanine (50 mmol) and Fmoc-L-lysine (50 mmol) to 1.0 ml water formed a suspension mixture. The mixture turned into clear sol when it was incubated in water bath to about 333 K. Then 10 mmol hemin chloride powders and 10 mmol histidine were mixed and dissolved in the peptides sol. After about 10 min, the artificial enzyme was formed under room temperature. Sodium alginate (1.1 wt%) solution was prepared by dissolving 1.1 g of sodium alginate powder in 98.9 ml of deionized water and filtered by 0.22 μm cellulous film. Mimetics were dropped on the surface of the GCE and allowed to dry at room temperature in dryer container for appropriate time and then covered with sodium alginate. When not in use, the electrode was stored in the refrigerator (4 °C). The free hemin modified electrode was prepared by dropping the same volumes of saturated hemin solution (pH 9.0 PBS) on the surface of the electrode. An HRP modified electrode was prepared by coating 2.5 μl 88 mg/ml HRP solution (the amount was optimized) on GC electrode. The other treatments are similar to that on the mimetic enzyme electrode. No water or buffer was added into the background solution deliberately. All the experiments were carried out in the ventilating closet for safety consideration. Before each mea-

surement, the background solution was saturated with nitrogen for about 5 min and a nitrogen environment was then maintained over the solutions in the cell during the detection.

2.4. Determination and recovery studies of organic peroxide in body lotion samples

About 0.5 g of body lotion sample was accurately weighed into a centrifuge tube and extracted with 1.0 ml ethyl acetate. Then, the organic layer was dried with anhydrous sodium sulfate for about 20 min. A background solution mimic was obtained by adding the same volume of water with body lotion samples to 1.0 ml ethyl acetate and treated the organic layer as above mentioned. In analysis, the standard solution with the tested sample solutions and the background solution mimic were alternatively injected into the cells. Recovery studies were carried out by adding 5, 10, 20 μl, 0.66 mol l^{-1} TBHP stock solutions into 1.0 mg body lotion samples, respectively, before pretreatment. TBHP was throughout employed as a standard.

3. Results and discussion

3.1. Characterization of supramolecular-hydrogel-based artificial enzyme

Molecular hydrogels have long been used as scaffolds in many applications, like tissue engineering (Silva et al., 2004) and biomaterials for wound healing (Yang et al., 2005). But it is the first application as matrices for enzymatic reactions in organic solvents (Wang et al., 2007b). This new kind of mimetic enzyme was prepared by immersing hemin and histidine which can efficiently enhance the catalytic activity by coordination of histidine to the Fe^{III} center in the hemin into the molecular hydrogel formed by two simple derivatives of amino acids: Fmoc-L-lysine and Fmoc-L-phenylalanine. After the formation of the gel, supramolecular hydrogels can localize hemin tightly within/around the nanofibers of the hydrogels and at the same time maintain some water molecular in the network of the hydrogels. Fig. 1 shows the UV–vis spectra of free hemin in pH 9.0 PBS and hemin in hydrogels with histidine. Free hemin in PBS (curve a) resulted in a Soret band at 386 nm which agrees with the reference as the presence of a mixture comprised of μ-oxo bihemin and the monomeric hemin hydroxide (Wang et al., 2007b). Therefore, the hemin dimers (385 nm) connected by μ-oxo bridges occupies the major part of the total amount of hemin in PBS. For comparison, UV–vis spectra of hemin mixed in the hydrogel with histidine (curve b) is also obtained and displays a Soret band at 404 nm which is similar to the absorption of the confined hemin in hydrogel (Wang et al., 2007b). Based on the consistent results with reference in UV–vis spectra, we conclude preliminarily that the artificial enzyme has been successfully synthesized in our experiments.

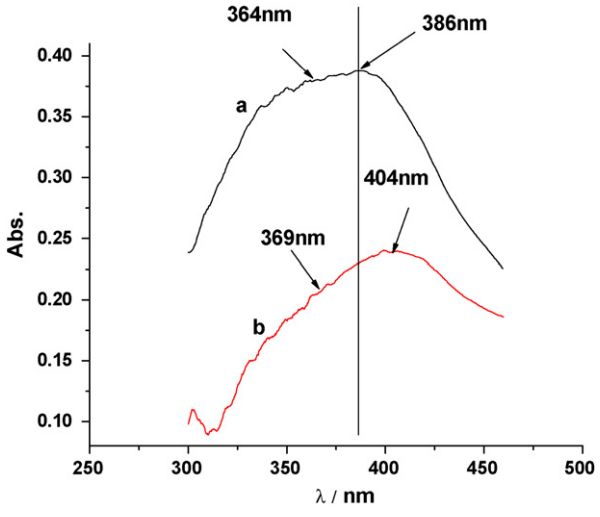

Fig. 1. UV–vis spectra of free hemin in pH 9.0 phosphate buffer solution (curve a), hemin in gel with histidine (curve b).

SEM measurement could provide us important information about the structure of supramolecular hydrogels. From the SEM imaging, nanofibers of the supramolecular hydrogel were evidently distinguished (Fig. 2(A) and (B)) which strongly supported the successful preparation of the supramolecular hydrogels. Since the SEM image was obtained from the artificial enzyme modified on glassy carbon slides under the same conditions with the modified electrodes, the well-kept shape of nanofibers indicated the unchanged structure of the artificial enzyme when it was applied on the surface of GCE. When covered with sodium alginate, the morphology of the nanofibers kept their original state (Fig. 2(C)) with slight changes on the surface of the nanofibers due to the cover of sodium alginate (Fig. 2(D)). Thus, from UV–vis spectra and the SEM image, we concluded the successful preparation of the artificial enzyme and consequent modification on GCE without changing enzyme's structure in our experiments.

3.2. The electrochemical behavior of the mimetic enzyme electrode

Fig. 3 shows the electrochemistry behavior of the mimetic enzyme modified electrode studied by cyclic voltammetry in the potential range of 0.3 to −0.75 V in THF containing 0.1 M TBAP. A pair of stable, well-defined and quasi-reversible redox peaks of the mimetic enzyme were observed in the graph (Fig. 3, curve c) with the formal potential ($E^{\circ\prime}$) of −0.251 V (vs. Ag/AgCl) which ascribes to the electrode process of hemin Fe(III)/Fe(II) couples entrapped in the mimetic enzyme. For comparison, free hemin and HRP modified electrodes were prepared. The free hemin modified electrode also showed a pair of redox peaks in the potential range of −0.15 to 0.75 V (Fig. 3, curve a), while the HRP modified electrode displayed a pair of asymmetric peaks and less evident redox peaks (see supplemental information, Fig. 1s). Both of the two pairs of peaks shifted negatively to a lower potential compared with mimetic enzyme modified electrode. The electrocatalytic ability to TBHP was further studied on the three kinds of modified electrodes. When adding 13.2 mM TBHP to tested solutions, there was a significant increase in the reduction peak and decrease in the oxidation peak on these modified electrodes, indicating a characteristic of the catalysis for the peroxide by the peroxidase. But the catalytic currents on hemin and HRP modified electrodes were much smaller than that on the mimetic enzyme electrode. For the same concentration of TBHP, the response on the mimetic enzyme modified electrodes was about 13 times of that obtained on the free hemin modified electrode (Fig. 3) and 3 times on HRP modified electrode (see supplemental information, Fig. 1s).

3.3. Optimization of the artificial enzyme modified electrode

As the matrix of supramolecular hydrogels can efficiently localize more hemin monomer due to its great ability of reducing the hemin dimerization, the biosensor shows better catalytic activity than free hemin modified electrode. However, the capacity of the matrix for immobilization of hemin is in certain range and over amount of hemin also results in the dimerization of hemin, and

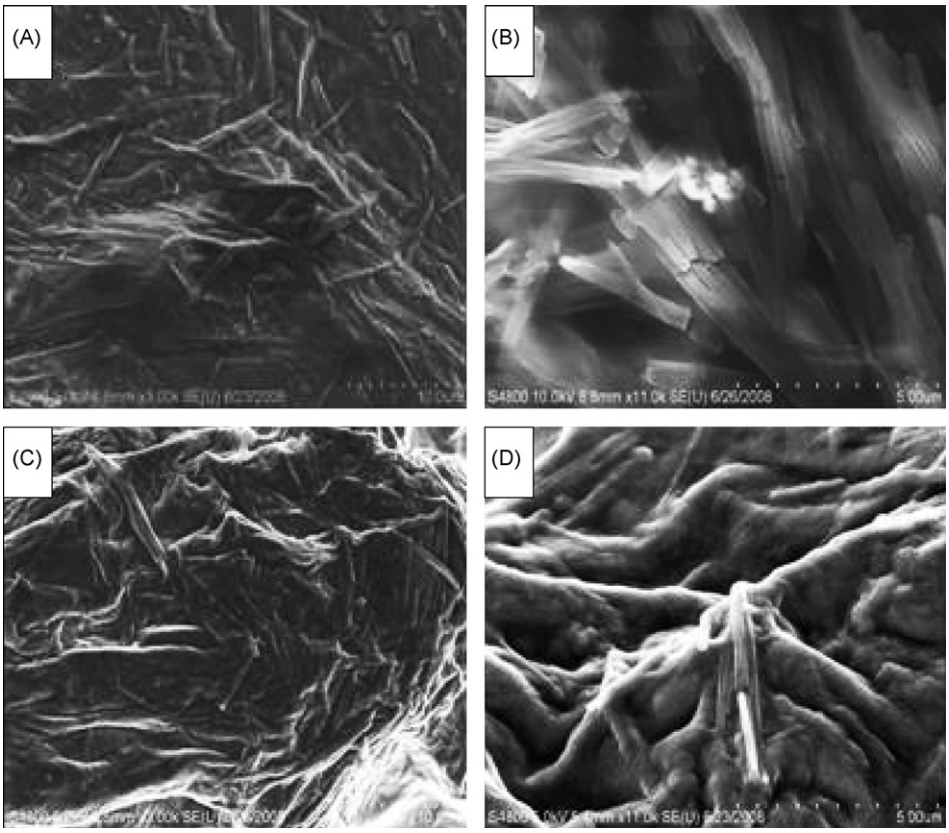

Fig. 2. SEM images of the supramolecular-hydrogel-based artificial enzyme modified glassy carbon slides before (A and B) and after (C and D) covering sodium alginate.

then the decrease of catalytic response for TBHP. Here, the optimal amount of hemin was 10 mmol accompanying with the same molar of histidine in the hydrogel.

To optimize the performance of the mimetic enzyme electrode, different amount of enzyme loading on the electrode was studied and the results showed that 2.5 μl was the best. It is believed that low enzyme amount is beneficial to receive high sensitivity to small variations of enzyme activities. But low concentration can also result in poor stability and reproducibility. Meanwhile, too high enzyme amount usually leads to longer response time, as the thick enzyme layer builds a diffusion barrier between substrates and electrode surface which limits the charge transfer. This could also be proved by our research—the response time increased with the increment of enzyme loading. Thus, we chose 2.5 μl as the amount of enzyme loading on the electrode.

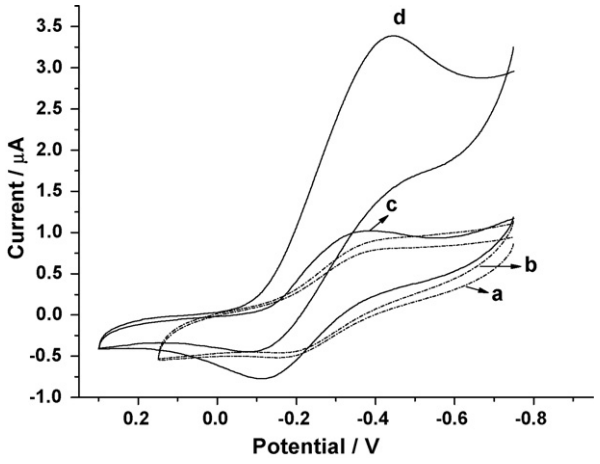

Fig. 3. Cyclic voltammograms of the modified electrode with free hemin (a and b) and mimetic enzyme (c and d) in the absence (a and c) and presence (b and d) of 13.2 mM TBHP in THF containing 0.1 M TBAP.

The artificial enzyme is based on the molecular hydrogels which contains and maintains certain amount of water in the skeleton and is likely to lose water, leading to the collapse of hydrogel structure when exposed in air for a long time. Retaining water in the hydrogels is one of the most important aspects to maintain the activity of artificial enzymes since we did not add buffer or water into the background solution before measurements. Thus, time for mimetic enzyme drying on the electrode was one of the key factors to determine the performances of the sensor. The optimal drying time is 30 min.

As sodium alginate is insoluble in most of the organic solvents (Mousty et al., 2001) and widely used for moisture retention in wound healing process (Qin, 2006), it was applied as a coating membrane to protect the artificial enzyme on the electrode surface. A bell shaped dependence of the catalytic current on the amount of sodium alginate loading was obtained with the maximum response at 3.5 μl. When the amount of sodium alginate is less than 3.5 μl, the artificial enzyme would be lack of the protection by sodium alginate which can not only retain water in mimetic enzymes but also protect mimetic enzymes from leaking to the organic solvents, during determinations. When the amount of the sodium alginate is over 3.5 μl, the response current decreased significantly. This is attributed to the increased membrane thickness, which would limit the diffusion of TBHP.

3.4. Effect of the organic solvent

The mimetic enzyme electrode was tested in different solvents in which the electrolyte TBAP can be readily dissolved. It was

Table 1
The response for 6.6 mM TBHP in different organic solvents containing 0.1 M TBAP.

Organic solvents	Response (μA)
Tetrahydrofuran (THF)	1.3 ± 0.06
DMF	0.87 ± 0.07
DMSO	0.8 ± 0.03
Ethyl acetate	0.41 ± 0.04
Dichloromethane	0.67 ± 0.03
Acetonitrile	0.43 ± 0.03
Chloroform	0.62 ± 0.07

reported that organic solvents had great influences on the catalytic properties of natural enzyme electrodes due to the effect of the hydrophilic–hydrophobic on the enzyme layer, the solvents viscosity and the solubility of electrolyte on the mass transport (Deng and Dong, 1995). Enzymes were easier to lose its hydration layer and consequently lose its activity in hydrophilic solvents. However, in our study, a relatively high response current was obtained in the so-called hydrophilic solvent of DMSO and DMF (Table 1). The reason may be that the artificial enzyme was less delicate than the natural one and thus was not so easy to denature and consequently lose the activity. At the same time, because of the protection of sodium alginate, the artificial enzyme could retain the essential amount of water in the skeleton and well kept its construction in the hydrophilic solvents. This was also consistent to the fact that a high and stable response was obtained while neither water nor buffer was added to the organic solvents beforehand. The water content in organic solvents can nearly be ignored compared with normal content of about 5% in most of the references (Morales et al., 2005; Cruz Vieira and Fatibello-Filho, 2000). The low catalytic current for the same concentration of TBHP in ethyl acetate can be ascribed to the poor solubility of TBAP in ethyl acetate. A high and stable response in THF, one of the hydrophobic solvents, was also obtained which cannot be achieved in previous reports (Cruz Vieira and Fatibello-Filho, 2000; Dong and Guo, 1994). Since THF, DMSO and DMF are excellent solvents for most of the analytes, the modified electrode will for sure broaden the utility of the OPEEs in detecting many kinds of substrates.

3.5. Sensing to TBHP

The electrocatalytic reduction of TBHP on the mimetic enzyme modified electrode was studied by amperometry. Fig. 4 shows the stepwise increase of the reduction current with the stepwise growth of the TBHP added to the organic solvent. The calibration curve gives a linear range of the concentration of TBHP from 6.6×10^{-5} to 1.27×10^{-2} M with a correlation coefficient of 0.9995, which was much wider than that obtained on the natural enzyme modified biosensors (Mulchandani et al., 1995; Gündoğan-Paul et al., 2002; Campanella et al., 2001b; Armada et al., 2004; Li et al., 1998; García-Moreno et al., 2001) and that received by the liquid chromatography method (Wada et al., 2003). At higher concentration of TBHP, the calibration curve tended to level off. The detection limit is 27 μM which is lower than that of other studies (Wada et al., 2003; Armada et al., 2004). The normal response time for analytes on OPEEs is around 1 min, and the least one was 18 s to reach 95% steady-state current (Wang and Dong, 2000). The long response time is due to the slow electron transfer rate in nonaqueous system, especially for the direct electron transfer between electrode surface and substrates. In our studies, the modified electrode needs only 10 ± 2 s to reach the 95% response, exhibiting a fast response to substrates.

3.6. Reproducibility and stability

The reproducibility of the organic phase sensor has also been explored by applying the mimetic enzyme modified electrodes in THF containing 0.1 M TBAP at a TBHP concentration of 1.32 mM. The relative standard deviation (R.S.D.) of the biosensor response is 1.1% for 10 successive measurements. The fabrication reproducibility of five sensors, independently prepared based on the same bare electrode, shows a fairly reproducibility with a relative standard deviation of 3.8% for the detection of 1.32 mM TBHP. The mimetic enzyme modified electrode can maintain 90% of its initial response in refrigerator (4 °C) in 1 week.

3.7. Determination of organic peroxides in body lotion samples

Oxidization of the long-chain lipids which occupy a large part in cosmetic products occurs during preservation and results in the formation of peroxides which leads to the irreversible deterioration of the cosmetics. Consequently, monitoring and detecting the peroxide in these products will be of great importance. Here, the artificial enzyme modified electrode was applied in determination of the organic peroxide in body lotion samples. Around 324 μg of the organic peroxides in body lotions were determined. Recoveries of 97.9–101.2% were obtained, indicating the absence of matrix influence on the determination (see supplemental information).

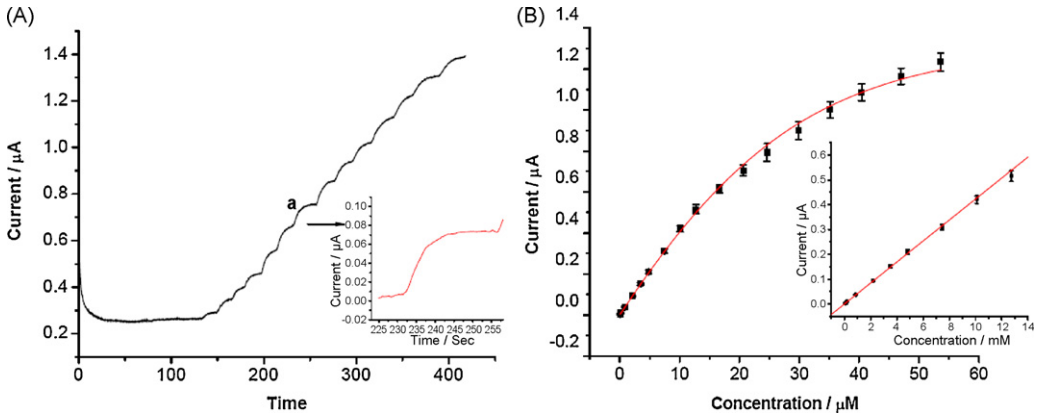

Fig. 4. (A) Steady-state current response of mimetic enzyme electrode on successive injection of TBHP into 1.0 ml stirring THF. Applied potential: −0.6 V. Inset: the magnification of the linear calibration curve. (B) Dependence of the electrocatalytic current on the concentration of TBHP (1); the magnification of one step in a (2) and (3).

4. Conclusions

A hemin-based artificial enzyme electrode in organic solvents is successfully prepared and used as a reagentless electrochemical sensor for the determination of TBHP. The skeleton of the artificial enzyme serves as an excellent carrier to immobilize greater amount of hemin and thus enhance the catalytic activity of hemin. A major advantage of this sensor is its simplicity and its fast, convenient application in both hydrophobic and hydrophilic solvents with a wide linear range, a low detection limit and a fast response for the analyte. The artificial enzyme modified electrode can be applied in determination of organic peroxides in real systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.10.009.

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