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Immobilization of horseradish peroxidase on nanoporous copper and its potential applications

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ABSTRACT

Nanoporous copper (NPC) with a pore size of 100–200 nm was prepared by simply dealloying $Al_{60}Cu_{40}$ alloy in a 5 wt.% HCl solution. The NPC was characterized by scanning electron microscopy and nitrogen adsorption techniques. Horseradish peroxidase (HRP) was immobilized on NPC by adsorption. Compared with free enzyme, the thermal stability of the immobilized enzyme was greatly improved due to the multiple attachments between the enzyme molecule and the NPC surface. After 2 h incubation at 50 °C, the immobilized HRP retained ca. 90% of the initial activity while only ca. 10% initial activity remained for the free enzyme. The interaction between HRP and the porous surface also made the K_m and K_{cat} values of the immobilized enzyme increase (from 0.43 to 0.80 mM) and decrease (from 8.1×10^3 to 2.2×10^3 min^{-1}), respectively. Based on the good electric conductivity and electrocatalytic activity of the NPC electrode, an electrochemical biosensor for *O*-phenylenediamine (OPD) was made. The calibration curve of the biosensor was linear from 0.5 μM to 14.5 μM OPD with a sensitivity of 0.37 μA μM^{-1}. The stability and reproducibility of the biosensor were also demonstrated to be good. When positioned at −0.45 V for 200 s, its current response toward 10 μM OPD remained ca. 80% of its initial value. For five HRP-loaded NPC electrodes, the relative standard deviation (RSD) of the current response toward 10 μM OPD was ca. 4.5%. All these results indicated that NPC was a good support for the HRP immobilization and its low price would facilitate its large-scale application.

1. Introduction

Immobilization of enzymes on solid supports is very important for practical applications due to the easy separation of enzymes from its reaction mixture and the reusability. To get an immobilized enzyme with high activity, the solid supports should be biocompatible and render the enzyme accessible to its cofactors and substrates when used for biosensing or biotransformation. In the past decades, porous inorganic materials especially porous silica have been widely used as an enzyme support due to its large specific surface area, negligible swelling, high stability and low price (Deere et al., 2002; Gao et al., 2009, 2010; Hudson et al., 2008; Rekuc et al., 2010; Takahashi et al., 2000). Recently, porous metal materials, as another kind of porous inorganic materials, have attracted much attention due to their special physicochemical properties.

At present, several methods have been developed for the fabrication of porous metal materials (Erlebacher et al., 2001; Shin et al., 2003; Stevens et al., 2002). The dealloying method is one of the most popular one. The so-called dealloying is a corrosion process during which one or more less-noble elements are removed from an alloy. Historically, dealloying is always correlated with corrosion, but recently it has been receiving renewed attention due to the fact that certain particular systems exhibit nanoporous evolution through dealloying (Erlebacher et al., 2001). Nanoporous metal materials such as nanoporous gold (NPG) obtained by dealloying Ag–Au alloy have been widely studied. Due to its unique mechanical, physical and chemical properties, NPG has been used in the areas of catalysis (Xu et al., 2007; Zeis et al., 2008), sensor/biosensor (Qiu et al., 2009c,d), surface-enhanced Raman scatting (Lang et al., 2009), and so forth. NPG has also been demonstrated to be a good support for enzymes (Qiu et al., 2009a,b; Shulga et al., 2007). Through the strong interaction between sulfur-containing amino acid (cysteine and methionine) on the enzyme surface and Au surface, the immobilized enzymes are very stable and retain high activity during repeated use. Due to the high electric conductivity and electrocatalytic activity of NPG, the enzyme-loaded NPG can also be used for the fabrications of electrochemical biosensor and biofuel cell (Qiu et al., 2008, 2009c). This feature of the enzyme-loaded NPG is an obvious

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advantage over the enzyme-loaded porous silica. In addition, the porous metal materials are very easy in preparation and recovery. One disadvantage of using NPG as an enzyme support is that gold is a precious metal and its high price may limit its large-scale application. As a metal, copper is much cheaper than gold. It has also been reported that copper has a good catalytic effect on the electrochemical reduction of some small molecules such as nitrate ions and organic nitrocompounds (Dima et al., 2003; Burke and Sharna, 2007). Therefore, the use of nanoporous copper (NPC) as a substitute for NPG for enzyme immobilization will be a good attempt. In previous work, we reported that monolithic NPC with uniform porous structure can be obtained by dealloying Al–Cu alloys with a composition range of 33–50 at.% (atom percentage) Cu (Qi et al., 2009). The bulky and free-standing NPC sample is very convenient in handling, fabrication of bioreactor/biosensor and also facilitates the study on the effect of the particle size of NPC on the immobilized enzyme.

Horseradish peroxidase (HRP) is one of the frequently encountered oxidoreductases. It has been used widely in bioreactor and biosensor. For example, HRP could be used to detect aromatic amines or phenolic compounds electrochemically via its covalent immobilization on gold nanoparticle modified porous silica electrode (Elyacoubi et al., 2006), physical entrapment in graphite and Teflon composite (Serra et al., 2003), or biospecific adsorption on concanavalin A modified gold electrode (Chen et al., 2008). However, the use of NPC as the substrate electrode has not been reported. In this work, we tried to demonstrate that NPC was a good support for the immobilization of HRP. HRP was immobilized on the NPC by adsorption. In addition to the immobilization conditions and the properties of the immobilized HRP, we also reported an electrochemical biosensor based on the immobilized HRP for the detection of *O*-phenylenediamine.

2. Methods

All experiments were done at least in triplicate and the results were presented as their mean value with the maximum relative deviation less than 8%.

2.1. Chemicals

Horseradish peroxidase (HRP, EC. 1.11.1.7, 250 U/mg), and *O*-phenylenediamine (OPD) were purchased from Sinopharm Chemical Reagent Co., Ltd. Coomassie brilliant blue G-250 was a product of Sanland-chem International Inc. Nafion (perfluorinated ion-exchange resin) was obtained from Sigma–Aldrich Co. All other regents were of analytical grade. Phosphate (50 mM)–citric acid (25 mM) buffer solution and triply distilled water were used throughout the experiments.

2.2. Preparation and characterization of NPC

Al–Cu alloy with a composition of 40 at.% Cu was prepared from pure Al (99.9%) and pure Cu (99.9%) as described in previous work (Qi et al., 2009). The Al–Cu alloy sheets with a thickness of 30–60 μm were dealloyed in a 5 wt.% HCl solution at ca. 85 °C for ca. 4 h until no H_2 bubbles were observed. Then the sample was rinsed six times with water and stored in a vacuum chamber. To study the particle size effects, NPC with two different particle sizes were used: Sample 1 was bulky pieces (ca. 6 × 6 mm); Sample 2 was fine NPC particles with a size of 0.02–0.5 mm (obtained by crushing Sample 1 with a pipette tip). The microstructure of NPC was characterized with a JEOL JSM-6700F field emission scanning electron microscope (SEM), which was equipped with an Oxford INCA x-sight energy-dispersive X-ray spectrometer (EDS) for compositional analysis. The surface area of the NPC was measured with Quadrasorb SI-MP (Quantachrome Instrument) using the BET method.

2.3. Immobilization of HRP on NPC

NPC (5 mg) was first kept in 2 mL of 1.5 mg mL^{-1} HRP solution at 4 °C for 20 h; then it was rinsed five times with 5 mL buffer (pH 5.8) to remove weakly adsorbed HRP on the outer surface and stored at 4 °C in a refrigerator under N_2 protection. The amount of HRP immobilized on NPC was calculated on the basis of the difference between the amount of HRP added and that recovered in the supernatant and washing buffer. HRP concentration was determined by the Bradford method, using bovine albumin as reference.

2.4. Determination of HRP activity

The activity of the free and immobilized HRP was determined spectrophotometrically on the basis of the absorbance change at 448 nm at 25 °C. For the immobilized HRP, 5 mg of HRP-loaded NPC was added into 3 mL of buffer solution (pH 5.8) containing 0.3 mM OPD and 1.2 mM H_2O_2. After 1 min stirring, the absorbance of the resulting supernatant was determined using a Shimadzu UV-2550 spectrophotometer. For the free HRP, 20 μL of free enzyme (0.5 mg mL^{-1}) was mixed in a cuvette with 3 mL of buffer solution (pH 5.8) containing 0.3 mM OPD and 1.2 mM H_2O_2, followed by monitoring the time dependent change in absorbance at 448 nm. The molar extinction coefficient of the oxidation product of OPD at 448 nm was 10.6 mM^{-1} cm^{-1}. One unit of activity was defined as the amount of enzyme required to have 1 μmol OPD oxidized in 1 min.

2.5. Enzyme kinetics

The initial reaction velocities of the HRP catalyzed oxidation of OPD at different concentrations of OPD (0.08–0.32 mM) were measured (HRP was saturated by H_2O_2). The concentrations of the free and immobilized HRP were 2 μg mL^{-1} and 1.67 mg mL^{-1} (the total weight of the immobilized HRP divided by the volume of the final solution), respectively. Other reaction conditions were the same as in Section 2.4. The Lineweaver–Burk plots were then made to obtain the apparent Michaelis–Menten constant of OPD (K_m) and the apparent maximum velocity (V_{max}). V_{max} was proportional to the enzyme concentration ([E]), as given in the following equation: $V_{max} = K_{cat}[E]$, where K_{cat} was an apparent rate constant with a unit of reciprocal time (min^{-1}).

2.6. Thermal stability and reusability

The thermal stability was evaluated by incubating free and immobilized HRP in the buffer solution (pH 5.8) at 50 °C. At given time intervals, 5 mg of immobilized enzyme (HRP-loaded NPC) in a separate cuvette or 5 μL of free enzyme from the same cuvette was taken out and left for 15 min to cool to the room temperature. The residual activity was then assayed at 25 °C. The initial activity value of HRP was taken as 100%. For repeated use, the immobilized HRP (5 mg) was taken out from the reaction mixture (pH 5.8) after 3 min reaction at 25 °C and rinsed three times with the buffer solution (pH 5.8). Then the immobilized HRP was added into the same but fresh reaction solution for the next use. Each time, the immobilized enzyme was taken out, and the reacted solution was immediately poured into a cuvette for absorbance measurement at 448 nm.

2.7. Leaching test

The immobilized HRP (on 5 mg NPC) was mixed with a leaching solution and incubated at 4 °C for 30 min. The leaching solution was 2 mL of the buffer solution (pH 5.8), the buffer solution containing 0.5 M NaCl, and the buffer solution containing 10% w/v polyethylene glycol, respectively. Then the immobilized HRP was removed and the remainder solution was tested using the Bradford method.

2.8. Preparation of HRP-modified NPC electrodes and electrochemical measurements

The HRP-modified NPC electrode was made by dropping 5 μL of Nafion solution (0.5 wt.%) on HRP-loaded NPC with a geometrical area of 7 mm^2. Then, the modified HRP-NPC electrode was left to dry at 4 °C and stored at 4 °C in a refrigerator when not in use. The formed Nafion film would avoid any enzyme leaching from the electrode. The HRP-modified Cu disk electrode with the same geometrical area was prepared as follows. First, 5 μL of HRP (2 mg mL^{-1}) was coated on the Cu disk surface and dried at 4 °C, then, 5 μL of Nafion solution (0.5 wt.%) was cast on the HRP-modified electrode surface and dried at 4 °C.

Electrochemical experiments were performed on a CHI 630C electrochemical workstation (CH Instrument Co., Shanghai, China) at room temperature. A three-electrode system was used, including a modified electrode as working electrode, a platinum wire as counter electrode and a saturated calomel electrode (SCE) as reference electrode. The test solutions were thoroughly deoxygenated with pure N_2 for about 25 min prior to each experiment. All potential values were versus SCE unless otherwise specified.

3. Results and discussion

The pore size of NPC was tunable to some extent by varying the etching time or the initial alloy composition or by applying thermal annealing after dealloying. In previous works, it has been demonstrated that nanoporous gold (NPG) with a pore size of >40 nm was suitable for the immobilization of laccase and acetylcholine esterase (Qiu et al., 2008; Shulga et al., 2007). A smaller pore size would be unfavorable for the free access of the biomacromolecules to the inside of the 3D ligament-pore structure during the immobilization and also slow down the mass transfer of the substrate and the product during the enzymatic reaction. To lower these negative effects by small pores, NPC with a pore size of ca. 100–200 nm was used in the present work for HRP immobilization. Moreover, it was considered that this NPC sample (obtained by dealloying Al–Cu (40 at.%) alloy) had a more uniform ligament-pore microstructure (Qi et al., 2009). The SEM image of the NPC (Qi et al., 2009) showed that it had a similar morphology to NPG obtained by dealloying Ag–Au alloy in previous work (Qiu et al., 2009a,b). EDS result showed that Al was completely removed from the Al–Cu alloy.

3.1. Immobilization of HRP on NPC

Compared with NPG, the big advantage of NPC was its low price, which would facilitate its large-scale application. However, the disadvantage of NPC was that Cu was not as stable as gold, especially under extreme pH condition and in the presence of O_2. Considering the fact that most enzymatic reactions were carried out at mild pHs, NPC may still be a good substitute for NPG for the present purpose. Before the immobilization of HRP, the stability of the NPC was first tested by immersing 5 mg of NPC in the buffer (2 mL) with a pH value varied from 2.0 to 8.0 under aerobic condition. For NPC in a buffer of pH 2.0, dissolution of NPC (~4 mM) was observed after 2-day storage, indicating the bare NPC was not stable at this condition. The dissolution of NPC decreased with the increase of the buffer pH value. NPC was relatively stable in buffers with pH within 5–8, and 2-day storage in the buffer only resulted in a slight dissolution based on the UV–Vis measurement. After long time storage (1 week), the color of the NPC turned dark from copper red, indicating the formation of Cu oxides. Interestingly, after the immobilization of HRP, the stability of NPC was greatly improved. No dissolution was observed for the HRP-loaded NPC (5 mg) in the buffers of pH from 2.0 to 8.0 after 2-day storage, and the color of NPC remained copper red even after one-week storage. It is well known that the modification of a metal (Fe, or Cu) surface with a layer of self-assembled small molecule could improve the corrosion stability of the metal (Zhou et al., 2008). Thus, the immobilized macromolecule HRP on the NPC surface may have a similar effect although, unlike the small molecule, the immobilized macromolecule layer might leave more vacancies on the metal surface due to their bigger size.

With regard to the immobilization mechanism, it is well accepted that $-NH_2$ and/or –S can react with the surface of Au and Cu, forming strong bond. The HRP used here has about 8 cysteine, 4 methionine and 7 lysine residues on or near its surface (data obtained from http://www.ncbi.nlm.nih.gov/). These sulfur-containing amino acid residues (cysteine and methionine) and $-NH_2$ containing amino acid residues (lysine) would facilitate the multipoint attachment of HRP on the NPC surface; in other words, some chemical adsorption occurred during the so-called physical adsorption process, which resulted in the least leaching of HRP. The leaching experiment showed that no protein was detected in the leaching solution of 50 mM buffer (pH 5.8) using the Bradford method. It was reported that salt/polyethylene glycol could readily desorb enzymes from the support if the enzymes were physisorbed (by electrostatic and/or hydrophobic interaction). In the present case, we found that NaCl and polyethylene glycol both had negligible effects on HRP desorption. This result indicated that chemical adsorption might contribute to the enzyme immobilization and the immobilization of HRP should be a monolayer on the NPC surface.

Our experimental results showed that 1 g NPC could have 8.8 mg HRP immobilized. The surface area of the NPC sample was ca. 3.2 $m^2\ g^{-1}$, which was obtained by N_2 adsorption–desorption (BET) method. The diameter of HRP was ca. 4.6 nm (assumed sphere) (Diaz and Balkus, 1996). If HRPs arranged themselves in an ideal monolayer, then 1 g of NPC could load ca. 13.7 mg of HRP (the molecular weight of HRP was ca. 44,000 Da). This value was larger than the experimental value, but it was reasonable since it was assumed during the calculation that there was no interaction between the HRP molecules, no blockage, etc. This also indicated that the surface of NPC was not fully occupied by the enzymes.

3.2. Properties of the immobilized HRP

In the past, we observed that when NPG with a pore size of 40–50 nm was used for laccase immobilization, the crushing of NPG was favorable for the immobilization of laccase on NPG in the same interval of incubation, indicating that the crushed sample (fine NPG particles) could enhance the accessibility of the enzyme to the inner surface of NPG (Qiu et al., 2009b). In the present case, however, it was found that, in the same time interval, almost the same amount of HRP was immobilized on the fine NPC particles and the bulky NPC (data not shown). The obvious difference from previous work should be attributed to the big pore size (100–200 nm) used in this work, i.e., the big pore size facilitated HRP into the inner pore and consequent immobilization. Thereby, no obvious difference in the amount of the immobilized HRP was observed after crushing. Similarly, the particle size of NPC also had no

obvious effect on the kinetic parameters of the immobilized HRP (Table 1). Compared with the free HRP, the K_m value of the immobilized HRP increased, i.e., the affinity of the immobilized HRP to the substrate OPD decreased. This might be due to the steric hindrance for the access of the OPD to the active site of HRP (the steric hindrance resulted from the complex inner pore structure of NPC) and/or the change of the active conformation of HRP after contact with the solid surface of NPC. The same reasons could also be used to explain the smaller K_{cat} value after the HRP immobilization. For the two immobilized HRP samples, similar K_m and K_{cat} values were observed. The similar K_m indicated that a similar enzyme conformation was obtained. The similar K_{cat} value indicated that the mass transfer inhibition for the two immobilized HRP was almost the same, i.e., the mass transfer was not further inhibited by the bulky size of NPC.

Over the pH range of 3.6–7.6, the immobilized HRP and the free one had similar pH-activity profiles with the same optimum value at pH 5.8. For the effect of temperature, the absorbance versus time curves at different temperatures indicated that at the initial stage (2 min), the slopes of both the immobilized HRP and the free HRP increased with the increase of temperature over 25–75 °C, but the linear range became narrower at high temperatures. For a given temperature, say 70 °C, the linear range of the immobilized enzyme was much longer than that of the free one (data not shown). This phenomenon was further demonstrated by the thermal stability experiment. As shown in Fig. 1, the activity of the free HRP decreased dramatically with the increase of the incubation at 50 °C. Two hours later, only ca. 30% of the initial activity was retained. Under the same conditions, however, the activity of the immobilized HRP decreased slowly and only ca. 10% of the initial activity was lost after 2 h incubation. The greatly improved thermal stability should be due to the multipoint attachment between HRP and the NPC surface (Kumar et al., 2010; Qiu et al., 2009a; Vaidya et al., 2008; Wang et al., 2001), which made the 3-dimentional structure of HRP more stable. The greatly improved thermal stability of the immobilized enzyme is very important for the practical industrial applications, in which usually high temperatures are needed to enhance the reaction rate, conversion and the solubility of some reactants. The repeated use decreased the activity of the immobilized HRP, but 65% of initial activity was retained after 5 cycles (Fig. 2). The decreased activity might be due to some leaching during the repeated washing and a long time contact with high concentrations of H_2O_2 (Qiu et al., 2009b). The storage stability of the immobilized HRP was also tested. After 1 month storage at 4 °C, ca. 90% initial activity was retained for the immobilized HRP. Under the same conditions, ca. 20% initial activity was lost for the free enzyme.

3.3. Electrochemical activity of the NPC-based electrode

As mentioned above, one of the big advantages of NPC over porous silica was its good electric conductivity. In this section, to explore the electrochemical applications of NPC, an OPD biosensor was fabricated based on the HRP-modified NPC electrode. Since H_2O_2 plays an important role in the HRP catalyzed oxidation reaction, the response of the electrode toward H_2O_2 was first tested.

Table 1
Kinetic parameters of free and immobilized HPR.

	K_m (mM)	V_{max} (mM min^{-1})	$K_{cat}/10^3$ (min^{-1})
Free HRP	0.43	0.35	8.1
Immobilized HRP (bulky NPC)	0.85	0.71	2.1
Immobilized HRP (fine NPC)	0.80	0.72	2.2

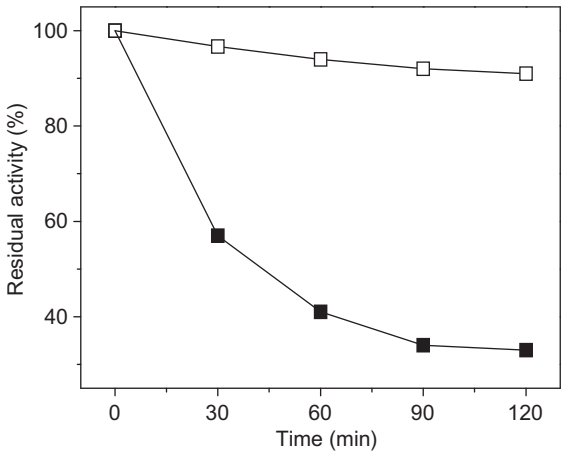

Fig. 1. Change of residual activity of HRP with incubation time at 50 °C: immobilized HPR (hollow); free HRP (solid).

Fig. 3a was the linear sweep voltammetry (LSV) curve of HRP-NPC electrode in the buffer (pH 5.8) containing 2.0 mM H_2O_2. The cathodic peak at ca. −0.6 V was due to the electro-reduction of H_2O_2 on NPC-based electrode. No reduction peaks was observed in the blank buffer solution. When 0.2 mM OPD was added into the H_2O_2 containing buffer, a new big reduction peak appeared at ca. −0.45 V (Fig. 3b). This peak should be due to the electro-reduction of the free radical of OPD generated by the HRP-catalyzed reaction. It is known that aromatic amines can be oxidized to free radical by peroxidases. The free radical can be electro-reduced at a certain potential and the current response is proportional to the concentration of aromatic amines in solution when H_2O_2 is in excess (Ruzgas et al., 1996). In comparison, at the HRP-Cu disk electrode (Fig. 3c), the reduction peak of the free OPD radical appeared at ca. −0.5 V. The positively shifted peak potential at the HRP-NPC electrode should be attributed to the improved electrocatalytic activity of NPC. As demonstrated elsewhere (Burke and Sharna, 2007; Dima et al., 2003; Pletcher and Poorabedi, 1979), Cu was one of the most effective metals to catalyze the reduction of nitrogen containing compounds (e.g. the conversion of $-NO_2$ to $-NH_2$). The active sites on Cu surfaces might be the defect sites (step edges) with lack of lattice stabilization energy (Burke and Sharna, 2007). In the present work, during the dealloying of the Al–Cu alloy, Al atoms were dissolved and Cu atoms left from the alloy/electrolyte interface would self-organize, forming islands (ligaments).

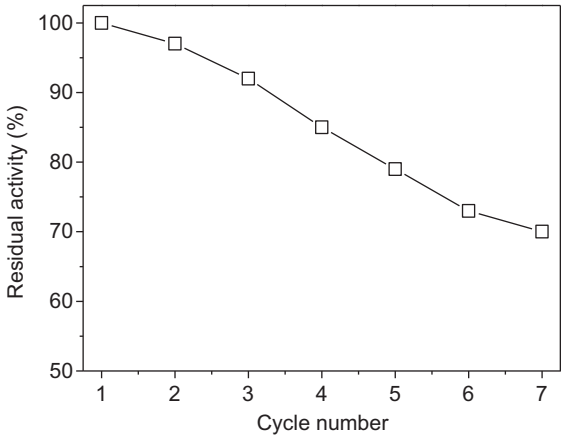

Fig. 2. Residual activity of the immobilized HRP after repeated uses at 25 °C and pH 5.8.

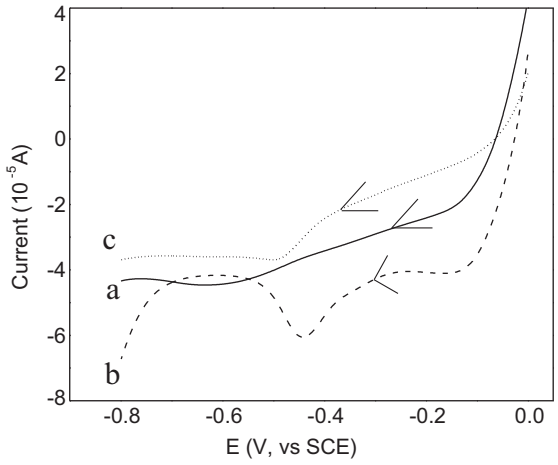

Fig. 3. Linear sweep voltammetry curves of HRP-NPC electrode (a and b) and HRP-Cu disk electrode (c) in a buffer solution (pH 5.8) containing 2.0 mM H_2O_2 (a), 2.0 mM H_2O_2 + 0.2 mM OPD (b and c).

Due to the extremely fast surface diffusion of Cu, many defect sites might appear together with the ligament formation. This may be the reason why the NPC-based electrode had an improved electrocatalytic activity toward the reduction of the OPD radical. A similar phenomenon has also been observed on the dealloying Ag–Au alloy (Zeis et al., 2008).

3.4. Amperometric detection of OPD

The current response of the HRP-NPC electrode toward OPD at −0.45 V was tested under stirring in the buffer (pH 5.8) containing 2.0 mM H_2O_2. The OPD was added successively with a concentration increment of 2.0 μM. The HRP-NPC electrode responded sensitively to each addition of OPD (curve *a* in Fig. 4). The current signal reached ca. 95% of the steady state current within 10 s. By contrast, the HRP-Cu disk electrode only had a negligible response toward OPD (curve *b* in Fig. 4). As shown in the inset of Fig. 4, the calibration curve obtained at the HRP-NPC electrode was linear

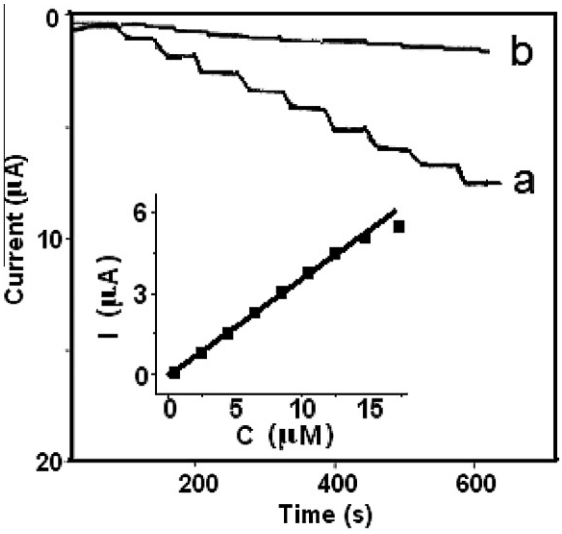

Fig. 4. Current–time curves of HRP-NPC electrode (a) and HRP-Cu disk electrode (b) toward OPD at −0.45 V in a buffer solution (pH 5.8) containing 2.0 mM H_2O_2 and different concentrations of OPD. The OPD was added successively with a concentration increment of 2.0 μM. Inset: calibration curves of OPD at HRP-NPC electrode.

(R = 0.997) from 0.5 μM to 14.5 μM OPD with a sensitivity of 0.37 μA μM^{-1} and a detection limit of 0.04 μM (S/N = 3). For five successive detection of 10 μM OPD on one electrode, the relative standard deviation (RSD) was 3.7%. For five HRP-NPC electrodes prepared in the same way, the RSD was 4.5% when detecting 10 μM OPD. The good reproducibility should be due to the good uniformity of the bicontinuous pore-ligament structure.

The stability of the fabricated biosensor was also tested. For continuous working under the potential of −0.45 V, the current response toward 10 μM OPD decreased to ca. 80% of its initial value after 200 s. The gradually decreased current response should be due to the gradual loss of active sites on the NPC-based electrode (Burke and Sharna, 2007). We observed that several cyclic voltammetry (CV) scans from −1.2 V to 0.6 V in the buffer (pH 5.8) could refresh the electrode. When not in use, the biosensor was stored in buffer (pH 5.8) under N_2 protection. To test the storage stability, the biosensor was used every day to measure its response toward 10 μM OPD for ten days. Before and after each test, the biosensor was refreshed by CV scans (−1.2 to 0.6 V) in the buffer solution. It was observed that the current response decreased slowly and ca. 92% current response was retained after ten days storage.

4. Conclusions

NPC with a pore size of 100–200 nm was obtained by dealloying $Al_{60}Cu_{40}$ alloy. By incubation with HRP aqueous solution, HRP was successfully immobilized on NPC surface. Due to the possible multiple attachments between sulfur-containing amino acids and the NPC surface, the immobilized HRP showed an excellent thermal stability and a good reusability. Based on the electric conductivity and electrocatalytic activity of the NPC, an electrochemical biosensor was also developed for the detection of OPD. The NPC-based biosensor was very sensitive to OPD when compared with HRP-modified Cu disk electrode due to the larger active surface area and higher electrocatalytic activity.

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