A nanoscaled Au–horseradish peroxidase composite fabricated by an interface reaction and its characterization, immobilization and biosensing†

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We develop a novel strategy for the biosensing application of hydrogen peroxide (H$_2$O$_2$) using nanoscaled Au–horseradish peroxidase (HRP) composite thin film synthesized by a liquid–liquid interface reaction. Through the interaction between Au nanoparticles and NH$_2$-terminated HRP, HRP is effectively combined with Au in the thin film. The nanocomposite membrane is extracted on the surface of the ITO electrode directly, retaining its bioactivity during the immobilization process, which can detect the substrate in situ. The immobilized HRP displays an excellent electrocatalytic response to the reduction of H$_2$O$_2$, with a fast amperometric response (within 5 s), wide linear range (7.9 μM to 3.6 mM), low detection limit (0.035 μM), and a good affinity ($K_{app}$ = 0.14 mM) to H$_2$O$_2$. The prepared biosensor also exhibits high sensitivity, good reproducibility and long-term stability. Furthermore, it can be successfully exploited for the determination of H$_2$O$_2$ released from living cells directly adhered on the modified electrode surface.

1. Introduction

Electrochemical biosensors have been recently applied to many fields including food analysis, biological analysis, environmental monitoring, and medical detection due to their intrinsic advantages such as high sensitivity, portability, relatively low cost, online detection, rapid response, and reusability. During the fabrication of biosensors, the strategy for immobilizing the active biomolecules is considered to be one of the crucial aspects, because biomolecules can be easily inactivated or released from the electrodes if they are not properly immobilized. Compared with the conventional immobilization methods of biomolecules such as physical adsorption, entrapment, cross-linking, covalent binding and the interface method, the liquid–liquid interface method is attractive owing to its inherent simplicity and low cost. Furthermore, it is an important means to generate self-assemblies of nanocrystals, providing a constrained environment for immobilization of biomolecules.

Gold nanoparticles (AuNPs) are one of the most useful metals which have been widely used in many sensing applications in recent years. AuNPs have the ability to enhance the electrode conductivity and facilitate the electron transfer. Especially, the small size and biocompatible ability of AuNPs were widely utilized as a base in the construction of various biosensors to promote the electron transfer of many proteins, such as horseradish peroxidase (HRP), heme globin, myoglobin, glucose oxidase and superoxide dismutase. In this work, we first use the liquid–liquid interface reaction to prepare the Au–HRP composite thin film for enzyme immobilization.

Macrophages (i.e. raw 264.7) are known to play an important role in host protection against a wide range of tumors and microorganisms. Being the first cells to participate in the immunological response, macrophages contribute to their role in host defense by phagocytosis, antigen presentation and the production of cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) involved in the destruction of pathogens. The main ROS and RNS produced in the macrophages are O$_2$•⁻, hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO). However, excess ROS and RNS production have been implicated in many inflammatory diseases.

In this work, a nanoscaled Au–HRP composite thin film is easily prepared through the interaction between assembled Au NPs and NH$_2$-terminated HRP at the toluene–water interface. The ITO electrode is chosen to extract the thin films up. Based on the ordered Au–HRP nanocomposite, a sensitive H$_2$O$_2$ biosensor is constructed. Compared with the conventional...
immobilization method, the biosensor has good sensitivity, reproducibility and long-term stability. The monitoring of \( \text{H}_2\text{O}_2 \) released from raw 264.7 macrophage cells using the proposed sensor is also demonstrated.

2. Experimental

2.1. Materials and reagents

HRP (E.C.1.11.1.7, 250 units per mg) was purchased from Sigma and used as received. \( \text{H}_2\text{O}_2 \) (30% W/V solution) solution was purchased from Beijing Chemical Reagent (Beijing, China). Indium tin oxide (ITO) glass (1.1 mm thickness, less than 100 \( \Omega \) resistance) was purchased from Conduc Optics and Electronics Technology. \( \text{H}_2\text{UCl}_4 \) was purchased from Shanghai Chemical Reagent Co., Ltd. Cetyltrimethyl ammonium bromide (CTAB) was purchased from Tianjin Chemical Reagent Institute. Hydrazine hydrate was purchased from Shanghai Ling Feng Chemical Reagent Co., Ltd. Triphenylphosphine (PPh3) was purchased from Sinopharm Chemical Reagent Co., Ltd. Toluene was purchased from Sinopharm Chemical Reagent Co., Ltd. Phosphate buffer solutions (PBS, 0.1 M) with various pH values were prepared by mixing stock standard solutions of \( \text{K}_2\text{HPO}_4 \) and \( \text{KH}_2\text{PO}_4 \) and adjusting the pH with \( \text{H}_3\text{PO}_4 \) or NaOH. All other chemicals were of analytical grade and were used without further purification. All solutions were made up with doubly distilled water.

2.2. Apparatus

The morphology and particle sizes of the samples were characterized by field emission scanning electron microscopy (FESEM) (LEO1530 VP) at an acceleration of 15 kV. Energy-dispersive spectroscopy (EDS) was performed on a microscope with a PV9100 scanning electron microanalyzer. The UV-vis absorption spectrum was obtained on a Varian Cary 5000 spectrophotometer. Circular dichroic (CD) measurements were performed on a CHI 660B electrochemical workstation (CH Instruments, USA). All electrochemical experiments were carried out in a cell containing 5.0 mL 0.1 M PBS at room temperature (20 \( \pm \) 2 \( ^\circ \)C) and the cell number was estimated using a hemocytometer.

2.3. Preparation of the nanoscaled Au–HRP composite

First, the AuNPs were prepared by a liquid–liquid interface method according to the previous report.\(^7\) Then, 0.75 mL of 2 mg mL\(^{-1}\) fresh HRP was dropwise added to the system and was uniformly spread onto the interface of the prepared AuNPs. After that, the system was kept at room temperature for 12 h. The HRP was chemisorbed into the prepared AuNP film by the interaction between AuNPs and NH\(_2\)-terminated HRP. The Au–HRP thin film was formed in the liquid–liquid interface as shown in Fig. S1.\(^\dagger\)

2.4. Preparation of the biosensor

A sheet of ITO (3 cm \( \times \) 0.5 cm) was first pretreated according to the literature.\(^3\) It was sonicated with diluted ammonia, absolute ethanol and deionized water for about 5 min, respectively. The ITO electrode was used to extract the Au–HRP thin films up to obtain Au–HRP modified ITO electrodes and washed with 0.1 M PBS (pH 7.0) to remove any unbounded enzyme from the electrode surface. After that, the Au–HRP modified ITO electrodes were dried in the desiccator. The Au/ITO and HRP/ITO electrodes were also prepared for the control experiments. The area of the working surface was approximately 5 \( \times \) 5 mm\(^2\). All of the prepared electrodes were stored at 4 \( ^\circ \)C prior to use.

2.5. Cell culture

Raw 264.7 macrophage cells were grown at 37 \( ^\circ \)C in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U mL\(^{-1}\) penicillin, and 100 mg mL\(^{-1}\) streptomycin in a 5% CO\(_2\) environment. After growing to 90% confluency, the cells were then washed three times with PBS (0.145 M NaCl, 1.9 mM NaH\(_2\)PO\(_4\), 8.1 mM K\(_2\)HPO\(_4\), pH 7.4) and the cell number was estimated using a hemocytometer.

2.6. \( \text{H}_2\text{O}_2 \) released from living cells

Raw 264.7 macrophage cells were incubated with zymosan (250 \( \mu \text{g mL}^{-1} \)) in DMEM complete medium in the presence of nitroblue tetrazolium (NBT). For cell adhesion, 0.5 mL of cells with a concentration of \( 2 \times 10^5 \) cells per mL was directly placed on the modified electrode for the electrochemical experiments. The adhered cells were fixed with 2% glutaraldehyde for 20 min at room temperature.

3. Results

3.1. Characterization of the Au–HRP nanofilm

X-ray diffraction (XRD) is used to study the nanostructured Au film and Au–HRP composite film on ITO substrates (Fig. 1). The diffraction peaks in the range of 10\(^{\circ} \times 2\theta < 85\(^{\circ}\) can be indexed to cubic structure Au (111), (200), (220), (311) and (222) (JCPDS card, no. 89-3697). The sharp peaks indicate that the product is well crystallized. In addition, some diffraction peaks of the In\(_2\text{O}_3\) (JCPDS card, no. 65-3170), marked with asterisks, are also observed, which come from the ITO substrates.

Fig. 2A and B show the FESEM images of the Au nanostructures at low (A) and high (B) magnification on ITO electrodes, respectively. The Au shows a chain-like nanostructure which is well distributed. By statistical analysis, the density of AuNPs on the ITO plate is about \( 8 \times 10^{10} \) atoms per cm\(^2\). Fig. 2C shows the FESEM image of the Au–HRP nanocomposite on ITO electrodes. Compared with the case in Fig. 2A, the product is
still well distributed, while the chain-like structure tends to aggregate. It results from the formation of the Au–HRP nanocomposite. HRP is immobilized orderly by the interaction between AuNPs and NH2-terminated HRP. Fig. 2D shows the FTIR spectrum of the AuNPs. The obvious absorption band at 3302 cm−1 and 1631 cm−1 can be assigned to the N–H and O–H vibration arising from amino groups and water absorption, respectively. The broad peak observed around 762 cm−1 is attributed to the N–H vibration. Therefore, the results forcefully indicate that the surface modification of amino groups is successful.

Fig. 3 shows the UV-vis spectra of Au, HRP and Au–HRP, respectively. Au does not show any absorption peaks (curve a). Comparing the two curves from Au–HRP (curve b) and HRP (curve c), the absorption band of HRP is located at 400 nm which is at the same position as the Au–HRP, suggesting that HRP is immobilized on Au and maintains its native structure. By calculation analysis, the density of HRP on Au is 1 mg mL−1.

In other words, such an immobilizing process does not destroy the structure of HRP and does not change the fundamental microenvironment of HRP.

Since CD spectroscopy is able to provide an insight into the structure and the conformation of the proteins/enzymes,36 CD spectroscopy is used to further characterize the structural integrity of HRP immobilized on Au. Fig. S2† shows the CD spectra of HRP and Au–HRP, respectively. The CD spectrum of HRP exhibits a negative peak at ca. 185 nm (curve a), which is similar to those of the other heme containing proteins.37 The position of Au–HRP (curve b) is almost the same as that from HRP. The similarities between the CD spectra of curves a and b in Fig. S3† indicate that the structure and the conformation of HRP remain after being immobilized on Au.

3.2. Electrocatalysis of immobilized HRP to reduction of H2O2

As demonstrated above, the Au–HRP composite thin film retains the structure and the conformation of HRP. Because the active HRP can amplify a weak signal and increase the detection ability of its target molecule H2O2, the electrocatalytic activity of the Au–HRP nanocomposite modified electrode toward H2O2 is investigated by cyclic voltammograms (CVs). Fig. 4 shows the CVs of different electrodes in 0.1 M pH 7.0 PBS at 100 mV s−1. Nanoscale Au modified ITO shows a curve similar to that of ITO. Thus Au is electroinactive in the potential window (figures not shown). The HRP modified ITO shows a small peak corresponding to the reduction of HRP (curve a'). While the Au–HRP modified ITO exhibits a couple of stable redox peaks which are attributed to the redox of immobilized HRP (curve c'), indicating that the presence of Au improves the direct electron transfer between the electrode and the immobilized HRP greatly. The anodic and cathodic peak potentials of the immobilized HRP are at −230 and −310 mV, respectively. The formal potential is −270 mV near the standard electrode potential of −220 mV (vs. SCE) of native HRP in solution,38 suggesting that most HRP molecules preserve their native structures after the immobilization process. Upon addition of H2O2 to the solution, the shape of the CV for HRP (curve b') and Au–HRP (curve d') both change with the increase of reduction currents displaying obvious electrocatalytic behaviors of the free and immobilized
HRP to the reduction of H₂O₂, respectively. The electrocatalytic responses are almost similar which shows that the enzyme activity is not affected after the immobilization process. No electrocatalytic current is observable at the Au modified ITO when H₂O₂ is added to pH 7.0 PBS (inset).

3.3. Optimization conditions for the biosensor

Some factors which influenced the performance of the sensor were investigated, including the pH of PBS and the applied potential (Fig. S3†). It showed that the current response increased from pH 5.0 to 7.0, and achieved a maximum value at pH 7.0, then decreased from pH 7.0 to 8.0, which was in accordance with the reported for soluble HRP. It meant that the existence of AuNPs did not change the optimal pH value for the bioelectrocatalytic reaction of the immobilized HRP to H₂O₂. In order to obtain maximum sensitivity and bioactivity, PBS with a pH of 7.0 was chosen as the buffer. The steady-state reduction current of H₂O₂ increased rapidly as the applied potential moved negatively from −0.2 to −0.4 V, which was due to the increased driving force for the fast reduction of H₂O₂ at a lower potential. Then the steady-state current decreased slightly when the applied potential was more negative than −0.4 V. The maximum current occurred at −0.4 V, which was selected as the working potential for amperometric detection of H₂O₂.

3.4. Amperometric response and calibration curve

The amperometric responses of the Au/ITO and Au–HRP/ITO upon successive additions of H₂O₂ to 0.1 M pH 7.0 PBS at an applied potential of −0.4 V are shown in Fig. 5. The Au/ITO showed a much smaller response to H₂O₂ (curve a in Fig. 5) than that of Au–HRP/ITO (curve b in Fig. 5) at the same H₂O₂ concentration. When an aliquot of H₂O₂ was added into the stirring buffer solution, the reduction current increased steeply to reach a stable value. The biosensor achieved the steady state current within 5 s. Such a fast response could be attributed to the following facts: H₂O₂ could diffuse to the enzyme freely since the HRP molecules were exposed to the surface of AuNPs. AuNPs made the HRP molecule immobilize orderly and were favorable to the orientation of the HRP molecules on the electrode surface during the process of electrocatalysis.

The linear range of H₂O₂ was from 7.9 µM to 3.6 mM (inset in Fig. 5) with a correlation coefficient of 0.9996 (n = 56) which was wider than 8.0 µM to 3.0 mM from HRP on the AuNP electrodeposited ITO electrode and 12.2 µM to 2.43 mM from HRP on a nano-Au monolayer modified chitosan-entrapped carbon paste electrode (CPE). The detection limit was 0.035 µM at 3σ which was much lower than 2 µM from HRP on the AuNP electrodeposited ITO electrode and 6.3 µM from HRP on an Au monolayer modified chitosan-entrapped CPE. At higher H₂O₂ concentrations, the response followed a typical Michaelis–Menten process. The apparent Michaelis–Menten constant (K_app), which indicates the enzyme–substrate kinetics, can be obtained from the Lineweaver-Burk equation. It was found to be 0.14 mM, which was smaller than 1.5 mM for the H₂O₂ biosensor fabricated by immobilizing HRP on the multiwalled carbon nanotube modified glassy carbon electrode (GCE) and 1.55 mM for the H₂O₂ biosensor fabricated by immobilizing HRP on nano-Au with the choline covalently modified GCE. It indicated that the prepared Au–HRP modified electrode exhibited a higher affinity for H₂O₂.

3.5. Selectivity, reproducibility and stability

To evaluate the selectivity of the proposed electrochemical biosensing system, a variety of relevant interfering species including O₂, OH⁻ and ONOO⁻ in the biological system was investigated for control experiments. 5.28%, 6.72% and 4.32% of cathodic current from O₂, OH⁻ and ONOO⁻ were observed, respectively (Fig. 6). Therefore, the as-prepared electrochemical biosensor would have good selectivity for monitoring H₂O₂ in biological samples.

The reproducibility of the current response of one enzyme electrode to 0.1 mM H₂O₂ was examined. The relative standard deviation (RSD) was 2.2% for seven successive assays. The electrode to electrode reproducibility was determined from the response to 0.1 mM H₂O₂ at five different enzyme electrodes...
with a RSD of 3.6%. The good reproducibility may be due to the fact that the AuNP made HRP molecules chemisorbed orderly and attach firmly onto the surface of AuNPs.

The long-time stability of the enzyme electrode was investigated over a 30 day period. When the biosensor was stored in the refrigerator at 4 °C and measured every 10 days, no obvious change was found in the response to 0.1 mM H₂O₂. The good long-term stability could be attributed to the fact that there were strong interactions between HRP molecules and the surface of AuNPs, thus, HRP molecules could be firmly immobilized on the surface of the Au and made the enzyme electrode stable.

3.6. Monitoring the H₂O₂ released from raw 264.7 cells

As mentioned above, the sensor exhibited a good sensitivity, selectivity, stability and reproducibility which were provided to be used in in vitro determination of H₂O₂ released from living cells. Fig. 7 shows the electrochemical response obtained at the Au–HRP modified ITO surface and towards rat 264.7 macrophage cells adhered on the electrode surface at an applied potential of −0.4 V in the absence (A) and presence (B) of phorbol-12-myristate-13-acetate (PMA), which was reported to generate H₂O₂ from cells.46 A much higher increased cathodic current was observed after the addition of PMA. The increased response was attributed to the reduction of H₂O₂ released from living cells. Thus, the sensor can monitor the H₂O₂ released from the cells. This result is consistent with the detection of H₂O₂ in PMA-treated macrophages by other reports.47–49

4. Conclusions

In summary, a novel method for immobilizing biomolecules on the surface of the electrode has been introduced. HRP has been successfully chemisorbed onto AuNPs that formed in the liquid–liquid interface via the interactions between AuNPs and HRP. The AuNPs provided the necessary conduction pathway and efficient electron tunneling. Furthermore, such modified HRP electrodes exhibited good electrocatalytic activity towards the reduction of H₂O₂. The resulting biosensor exhibited fast amperometric response, low detection limit, wide linear range to H₂O₂, high sensitivity, good reproducibility, and long-term stability. This approach provided a novel and simple method for developing the immobilization method and a new class of electrochemical biosensor for H₂O₂. More interestingly, it could be successfully exploited for the determination of H₂O₂ released from living cells which was expected to have potential application in cellular biology.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China for the project (nos 21475062, 21175069, 21171096 and 21271105), Foundation of the Jiangsu Education Committee (11KJA150003), and Research Fund for the Doctoral Program of Higher Education of China (20113207110005). We appreciate the financial support from the Priority Academic Program Development of Jiangsu Higher Education Institutions and the Program for Outstanding Innovation Research Team of Universities in Jiangsu Province.

Notes and references
