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ORIGINAL PAPER

Direct electrochemical behavior of hemoglobin at surface of Au@Fe_3O_4 magnetic nanoparticles

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Abstract Nanoparticles (NPs) consisting of an Fe_3O_4 core and a thin gold shell (referred to as Au@Fe_3O_4 NPs) were self-assembled on the surface of a glassy carbon electrode modified with ethylenediamine. Following adsorption of hemoglobin, its interaction with the NPs was studied by UV–Vis spectroscopy, electrochemical impedance spectroscopy, and cyclic voltammetry. Stable and well-defined redox peaks were observed at about −350 mV and −130 mV in pH 6.0 buffer. The modified electrode was used as a mediator-free sensor for hydrogen peroxide (H_2O_2), with a linear range from 3.4 μM to 4.0 mM of H_2O_2, and with a 0.67 μM detection limit (at an S/N of 3). The apparent Michaelis-Menten constant is 2.3 mM.

Keywords Au@Fe_3O_4 nanoparticles · Hemoglobin · Hydrogen peroxide · Direct electrochemistry

Introduction

With the development of nanotechnology, more and more new nanomaterials with good bicompatibility and electroconductivity have been synthesized and widely used in electrochemical biosensor. Among them, shell-core magnetic Au@Fe_3O_4 NPs as specially immobilizing carrier of biomolecules have aroused great interest [1]. The inner Fe_3O_4 core with outer shell of gold not only improves its chemical stability and dispersibility in solution but also provides sites for chemical functionalization through the attachment of thiolated molecules, which can form self-assembled monolayers [2, 3]. In addition, the gold shell is also helpful to increase the biocompatibility and electroconductivity of Fe_3O_4 NPs as well as enlarge the surface area, which is good for immobilizing more biomolecules. Therefore, Au@Fe_3O_4 NPs hold much promise for biosensor applications. To the best of our knowledge, no work has been reported on the direct electron transfer of proteins immobilized on its interfaces.

Hemoglobin (Hb) is an ideal model molecule for study of electron transfer reactions of heme proteins because of its commercial availability, moderate cost and a known and documented structure [4]. Studies of the electrochemical behavior of Hb are important for a fundamental understanding of its biological activity. Unfortunately, it is usually difficult to realize electron transfer between Hb and conventional electrodes due to its electroactive center buring in the large three dimensional structure [5, 6]. Many efforts have been made to improve the electron transfer of Hb by using mediators and promoters especially by modifying electrode with desirable matrix such as surfactants [7, 8], nanoparticles [9–11] and polymers [12, 13].

In this work, Au@Fe_3O_4 NPs were firstly prepared and further used to immobilize Hb on the electrochemically pretreated glassy carbon electrode (PGCE) using ethylenediamine as a cross-linker. NPs can provide large surface area, superior conductivity and favorable microenvironment for retaining the bioactivity of Hb and facilitate its direct electron transfer. The electrochemical behavior of Hb was

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discussed in detail, and an effective H_2O_2 biosensor was constructed.

Materials and methods

Chemicals

Hb (~90%, bovine blood), ethylenediamine and $LiClO_4$ were purchased from Sigma (St. Louis, MO http://www.sigmaaldrich.com/united-states.html) and used without further purification. Gold chloride ($AuCl_3 \cdot HCl \cdot 4H_2O$, Au% >48%), $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ were purchased from Shanghai No.1 Reagent Factory. H_2O_2 solution was freshly prepared before being used. The buffer solutions (PBS, 0.1 M) were prepared from Na_2HPO_4 and NaH_2PO_4, and the pH adjusting was regulated with H_3PO_4. Other chemicals were of analytical grade. All solutions were prepared with twice-distilled water.

Apparatus and methods

Electrochemical measurements were performed with a CHI 660 electrochemical workstation (CH Instruments Co., USA) using the modified glassy carbon electrode (GCE, Φ=3.0 mm) as the working electrode, a platinum wire as the counter and a saturated calomel electrode (SCE) as reference electrode. Amperometric experiments were carried out in a stirred system by applying a potential of −200 mV to the working potential. Electrochemical impedance spectroscopy (EIS) experiments were performed at a potential of 0.17 V within the frequency range from 10^{-2} to 10^5 Hz in 0.1 M KNO_3 containing 5.0 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (1:1). An alternating current voltage of 5 mV was applied and 12 data points per frequency decade chosen to be equidistant on the logarithmic scale were recorded. The UV–vis spectrum was recorded on an UV-2450 spectrophotometer (Shimadzu, Japan). The morphology of NPs was investigated using a transmission electron microscope (TEM) (JEOL-1210, Japan).

Synthesis of Au@Fe_3O_4 NPs

Fe_3O_4 core NPs were synthesized according to reported method [14] with minor modifications (see the electronic supplementary material (ESM)). Au-shell coating was performed by reduction of Au^{3+} on the surface of Fe_3O_4. Briefly, solution of 0.1% $AuCl_3 \cdot HCl$ was added to a three-necked flask, and heated to boiling with vigorous stirring. Then a certain amount of Fe_3O_4 was added to the boiling solution of $AuCl_3 \cdot HCl$. The reaction was continued with vigorous stirring at 100°C under reflux for about 30 min before being cooled to room temperature. The obtained colloidal solution was isolated in a magnetic field, and the supernatant was removed from the precipitate by decantation. Finally, Au@Fe_3O_4 NPs were washed thoroughly and dispersed in twice-distilled water.

Electrode modification

GCE was firstly polished with abrasive paper and then with alumina slurry, followed by ultrasonically cleaned in ethanol and water and dried in air. PGCE and ethylenediamine modified PGCE were obtained according to our previous report [15]. The resulting electrode was soaked in water to remove the physically adsorbed ethylenediamine. Then, it was dipped into the Au@Fe_3O_4 NPs for 10 h and the 10 mg mL^{-1} Hb solution (pH 6.0 PBS) for 20 h at 4°C. The resulting Hb/Au@Fe_3O_4 modified electrode was washed with water and stored in pH 7.0 PBS at 4°C for use.

Results and discussion

Characterization of Au@Fe_3O_4 NPs

Transmission electron microscopy (TEM) was performed to observe the microstructure of the NPs (see the Fig. S1 in the ESM). It is clear that the average diameter of Fe_3O_4 was about 15 nm (Fig. S1A). After modification of the Fe_3O_4 with Au shell, the resulted shell-core Au@Fe_3O_4 NPs retained the spherical structure with the average diameter of 30–40 nm (Fig. S1B). The thickness of the Au shell was determined to be 15–25 nm. The result showed that a layer of Au has been coated uniformly on the surface of the Fe_3O_4, which can contribute to enhance the dispersibility of magnetic NPs.

UV–vis spectroscopy provided an indirect piece of evidence supporting the formation of Au@Fe_3O_4 core-shell morphology. Figure 1 shows a typical set of UV–vis

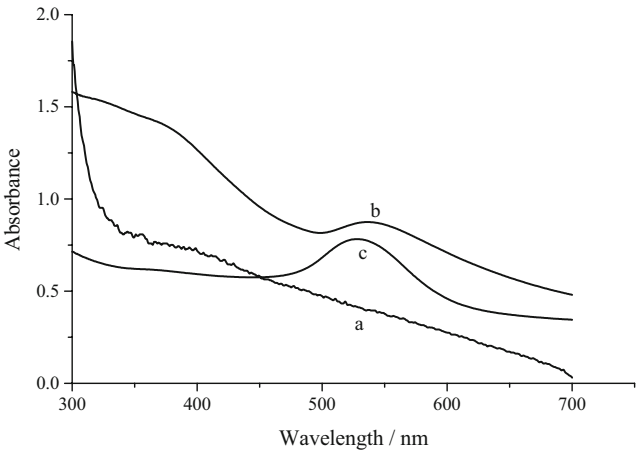

Fig. 1 UV–vis spectra of synthesized (*a*) Fe_3O_4, (*b*) Au@Fe_3O_4, (*c*) Au NPs

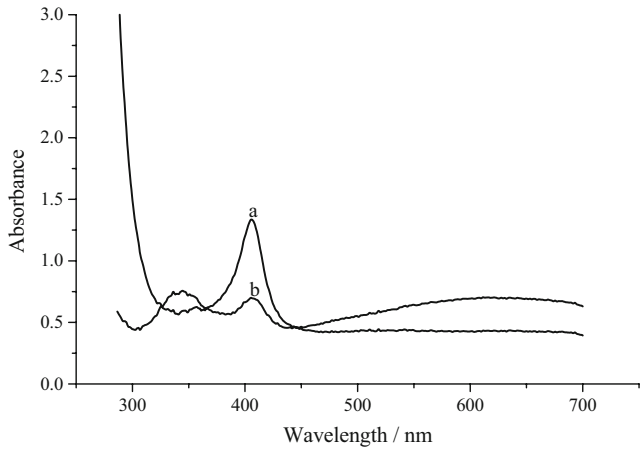

Fig. 2 UV–vis spectra (*a*) in pH 7.0 PBS containing 0.5 mg mL^{-1} Hb, (*b*) at a Hb/Au@Fe_3O_4/ethylenediamine modified pretreated ITO

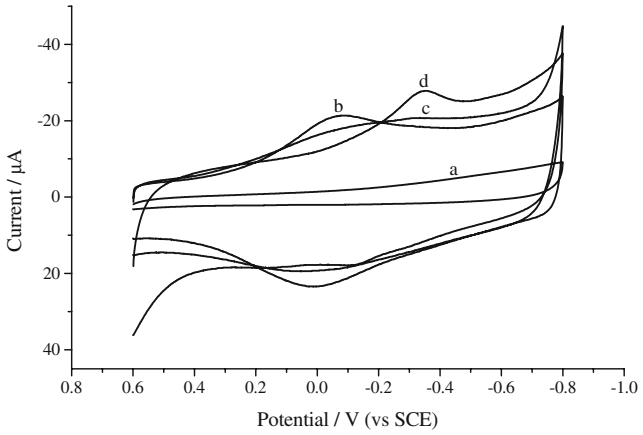

Fig. 4 CVs of (*a*) bare GCE, (*b*) PGCE, (*c*) Au@Fe_3O_4/ethylenediamine modified PGCE and (*d*) Hb/Au@Fe_3O_4/ethylenediamine modified PGCE (50 mV s^{-1}) in pH 6.0 PBS

spectra comparing Fe_3O_4 and Au@Fe_3O_4 NPs. There were no significant adsorption peaks in the visible region for Fe_3O_4 NPs (Fig. 1a), while Au@Fe_3O_4 NPs display a peak around 536 nm (Fig. 1b), showing a small red-shift in comparison with pure Au NPs of 520 nm (Fig. 1c) [16]. Previous studies showed that shell-core Au@Fe_3O_4 NPs displayed red-shift which was dependent on the thickness of Au shell [17]. The results indicated the formation of Au shell on the surface of Fe_3O_4 NPs.

Fabrication and characterization of Hb/Au@Fe_3O_4 NPs modified electrode

Many studies have revealed that after electrochemical pretreatment, the surface of the carbon electrode can be oxidized and thus contain various kinds of oxygenous groups [18, 19], which will improve the electrode surface of the reaction [20]. Some compounds including functional groups such as -NH_2 and -SH can be chemisorbed on the surface of glass or Au and so on to form monolayer. The other end of the molecule monolayer was -NH_2 and -SH, which can form strong covalent bond with gold nanoparticles [21, 22].

Here, Au@Fe_3O_4 NPs were firstly immobilized on ethylenediamine modified PGCE through Au–N bond. Then, Hb was assembled through the interaction of Au out of Au@Fe_3O_4 and -NH_2 in Hb molecules. Thus, Hb can be assembled on the surface of the modified electrode.

UV–vis spectroscopy is an effective means for monitoring the possible change of the Soret absorption band in the heme group region. The band shift may provide information about possible denaturation of heme proteins, especially that conformational change around the heme region. The UV–Vis spectra of Hb in pH 7.0 PBS resulted in a

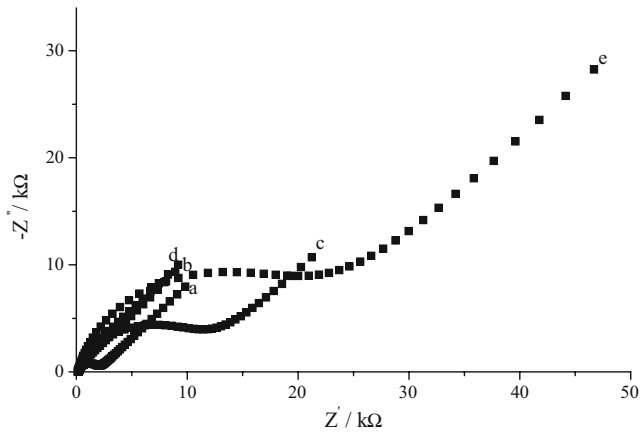

Fig. 3 The electrochemical impedance spectroscopy (EIS) of (*a*) GCE, (*b*) PGCE, (*c*) ethylenediamine modified PGCE, (*d*) Au@Fe_3O_4/ethylenediamine modified PGCE and (*e*) Hb/Au@Fe_3O_4/ethylenediamine modified PGCE in 0.1 M KNO_3 solution containing 5.0 mM $Fe(CN)_6^{3-}$/ $Fe(CN)_6^{4-}$ (1:1)

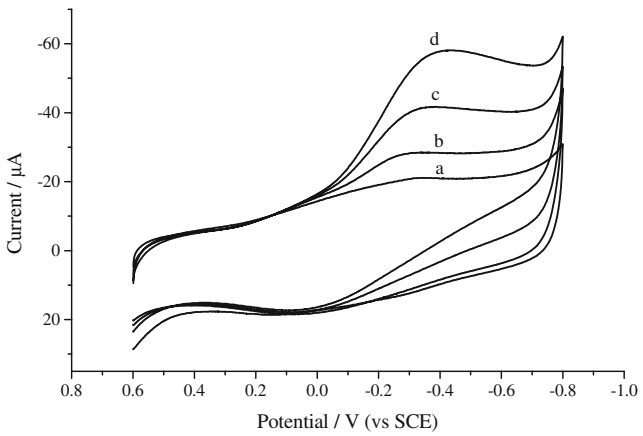

Fig. 5 CVs of Hb/Au@Fe_3O_4/ethylenediamine modified PGCE in pH 6.0 PBS (50 mV s^{-1}) (*a*) in the absence of H_2O_2, (*b*), (*c*) and (*d*) in the presence of 9.3×10^{-4} M, 2.0×10^{-3} M, 3.2×10^{-3} M H_2O_2, respectively

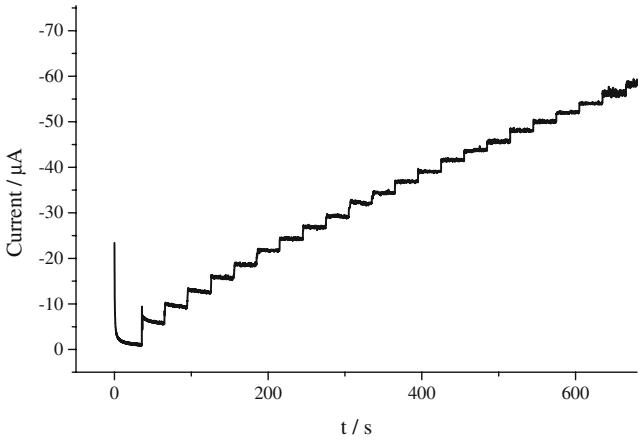

Fig. 6 Amperometric responses with successive additions of 6.5×10^{-5} M H_2O_2 in pH 6.0 PBS

Soret band at 406 nm (Fig. 2a), while shifted to 407 nm at Au@Fe_3O_4 surface (Fig. 2b). The band position of Hb was slightly moved, indicating that the microenviroment of Hb hosted was mimic to its own native system and thereby no significant denaturation happened on Au@Fe_3O_4 film. The slight shift in Soret band may be due to the interaction between Au@Fe_3O_4 NPs and Hb biomolecules.

EIS was often used to monitor the assembly process. In EIS, the semicircle part at higher frequencies corresponds to the electron-transfer limited process, its diameter equals the electron transfer resistance, $R_{et,}$ which exhibits the electron transfer kinetics of the redox probe at the electrode interface. To understand clearly the electrical properties of the as prepared electrodes/solution interfaces, the Randles equivalent circuit was chosen to fit the obtained impedance data. Figure 3 displays the EIS observed upon the changes of surface-modified process. Compared with the bare GCE (Fig. 3a) and PGCE (Fig. 3b), the Nyquist diameter of the ethylenediamine modified PGCE (Fig. 3c) increased dramatically, which suggested that the ethylenediamine coated on the electrode can obstruct the electron transfer of the redox probe. After Au@Fe_3O_4 NPs were immobilized, the semicircle diameter was obviously reduced (Fig. 3d), indicating that Au@Fe_3O_4 NPs play an important role similarly to a conducting wire, which makes it easier for the electron transfer take place. After adsorption of Hb, an obvious increase again in the interfacial resistance was observed (Fig. 3e). The impendence change of modification process showed that Hb had been immobilized on the electrode surface.

Direct electrochemistry of Hb immobilized on Au@Fe_3O_4 NPs

The electrochemical behavior of Hb at surface of Au@Fe_3O_4 was studied by cyclic voltammetry. Figure 4 shows the CVs obtained from different modified electrodes in pH 6.0 PBS. No peak was observed at bare GCE (Fig. 4a), after being electrochemically pretreated, PGCE displayed a pair of nearly symmetrical peaks (Fig. 4b). When Au@Fe_3O_4 NPs assembled on the ethylenediamine modified PGCE, a pair of peaks at PGCE almost disappeared (Fig. 4c), while a couple of stable and well-defined redox peaks was observed after combining with Hb with potentials of E_{pc}=−0.35 V and E_{pa}=−0.13 V (Fig. 4d). The formal potential ($E^{\circ'}$) was −0.24 V for Hb. These results revealed that the presence of Au@Fe_3O_4 may be provide a friendly microenvironment for immobilizing Hb, greatly increased the biomolecules loading and decreased the electron transfer distance between the electroactive center of Hb and underlying electrode, which in turn contributed to the realization of the direct electrochemistry of Hb.

With scan rate increased from 40 to 350 mV s^{-1}, the redox peak currents of Hb increased linearly and potentials almost unchanged, showing a surface-controlled process [23]. It also suggested that all the electroactive sites of Hb (Fe^{3+}), are converted to Hb (Fe^{2+}) on the forward cathodic scan with full conversion of Hb (Fe^{2+}) to Hb (Fe^{3+}) on the reverse anodic scan [24].

Table 1 The performance of electroanalytical methods for sensing H_2O_2 using Hb

Methods[a]	Linear range (M)	LOD (M)	K_m (mM)	Stability	Ref.
Hb-TNT/C/GCE	1.0×10^{-6}–1.0×10^{-4}	9.2×10^{-7}	0.088	95% retained after 2 weeks	[5]
Hb-chitosan/$CaCO_3$/GCE	3.7×10^{-5}–8.3×10^{-4}	8.3×10^{-6}	0.75	Not reported	[11]
Hb/P123/PG	1.0×10^{-6}–5.0×10^{-4}	5.0×10^{-7}	0.51	90% retained after 2 weeks	[12]
Hb-PCL/GCE	2.0×10^{-6}–3.0×10^{-5}	6.1×10^{-6}	0.037	90% retained after 2 weeks	[13]
Hb/titanate/PG	2.0×10^{-5}–3.2×10^{-3}	8.0×10^{-6}	Not reported	Not reported	[26]
Hb-V_8SH/Au	1.0×10^{-5}–1.2×10^{-4}	2.5×10^{-7}	Not reported	90% retained after 2 weeks	[27]
Hb/IL/CILE	1.0×10^{-4}–5.0×10^{-3}	4.0×10^{-5}	7.5	quite stable after a month	[28]
Proposed biosensor	3.4×10^{-6}–4.0×10^{-3}	6.7×10^{-7}	2.3	92% retained after 1 week	

[a] TNT/C: Carbonized TiO_2 nanotubes; P123: PEO-PPO-PEO; PCL: poly(ε-caprolactone); V_8SH: thiolated-viologen; IL: octylpyridinium chloride; CILE: carbon ionic liquid electrode

According to Laviron's equation [25]:

$$i_p = \frac{nFQv}{4RT} \quad (1)$$

Q is the quantity of charge (C), calculated from the peak area of the voltammogram. The symbols n, i_p, F, R and T have their usual meanings. From the slope of the $i_p \propto v$, n was calculated to be 0.86. Therefore, the redox of Hb on Au@Fe_3O_4 is a single electron transfer reaction.

Bioelectrocatalysis of the Hb immobilized on Au@Fe_3O_4 NPs toward H_2O_2

When H_2O_2 was added to the PBS, an increase in the reduction peak was observed with the decrease of the oxidation peak for Hb (Fig. 5). The reduction peak current increased with increasing concentration of H_2O_2, indicating that the immobilized Hb exhibited excellent bioelectrocatalytic activity toward the reduction of H_2O_2.

Figure 6 shows the chronoamperometric curve of the resulting electrode upon successive additions of H_2O_2 to PBS (pH 6.0) under the optimized conditions. The biosensor responded rapidly to the substrate increase and achieved 95% of steady-current in 10 s. There is a linear relation of the current with concentration of H_2O_2 from 3.4×10^{-6} to 4.0×10^{-3} M, the linear regression equation was $-\mathrm{I(A)} = 2.2\times10^{-6} + 0.026(\mathrm{H_2O_2})(\mathrm{M})\left(\mathrm{R}^2 = 0.9992\right)$, with a detection limit was estimated as 6.7×10^{-7} M (at an S/N of 3). The performance of the proposed biosensor was compared with other biosensors, as shown in Table 1.

The apparent Michaelis-Menten constant (K_m), which gives an indication of the enzyme-substrate kinetics, could be obtained from the Lineweaver-Burk equation [29]:

$$\frac{1}{I_{SS}} = \frac{K_m}{I_{max}} \cdot \frac{1}{C} + \frac{1}{I_{max}} \quad (2)$$

Here, I_{ss} is the steady state current after the addition of substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated substrate conditions. K_m value for Hb/Au@Fe_3O_4 modified electrode was found to be 2.3 mM. The lower value of K_m indicated that Hb immobilized at surface of Au@Fe_3O_4 exhibits a high biological affinity to H_2O_2.

Stability and reproducibility

The relative standard deviation (R.S.D.) was 1.6% for 10 successive measurements at 0.5 mM H_2O_2, indicating a good precision. Moreover, a series of five electrodes prepared in the same manner were also tested and the R.S.D. observed was only 3.8%. When not in use, the modified electrode was stored in pH 7.0 PBS at 4°C, after 1 week, the response current retained more than 92% of its original response. The long-term stability may be attributed to the strong covalent interaction between ethylenediamine, Au@Fe_3O_4 and Hb which is not affected by the changes such as the solvent pH, solution concentration, ionic strength, temperature and so on.

Analytical application

By using a standard addition method, the recovery experiment was performed by adding different concentrations of H_2O_2 in 0.1 M pH 6.0 PBS. The results were satisfactory with the recovery between 96.6~107.5% (see Table S1 in the ESM).

Conclusions

Au@Fe_3O_4 NPs, which possessed the advantages of chemical stability, magnetism and ease of functionalization, were synthesized and further used to immobilize Hb. The properties of Hb assembled at Au@Fe_3O_4 were characterized by both spectroscopic and electrochemical techniques. Due to the good conductivity and biocompatibility, Au@Fe_3O_4 NPs can significantly promote the direct electron transfer of Hb with the underlying electrode. At the same time, the immobilized protein retained its biological activity and showed excellent bioelectrocatalytic activity to the reduction of H_2O_2. We believe that the method presented here can be extended to study the direct electron transfer of other proteins and construct third generation biosensors.

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