A novel electrochemical immunosensor based on nonenzymatic Ag@Au-Fe₃O₄ nanoelectrocatalyst for protein biomarker detection

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Abstract

A hybrid nanostructure of Fe₃O₄ nanospheres and Ag@Au nanorods prepared by polydopamine coating was utilized as nanoelectrocatalyst to construct a novel sandwich-type electrochemical immunosensor. Ag@Au-Fe₃O₄ nanohybrid modified electrode exhibited much better electrocatalytic activity toward the reduction of hydrogen peroxide than Fe₃O₄ nanospheres or Ag@Au nanorods due to the synergetic catalytic effect. The immunosensor was prepared by immobilizing the capture antibodies on the amine-terminated nanocomposite of carbon nanofibers-chitosan, whilst the trace tag was prepared by loading detection antibodies on the Ag@Au-Fe₃O₄ nanocomposite. After the parameter optimization, the amperometric signal increased linearly with human IgG concentration in the broad range of 0.1 pg mL⁻¹ to 5 µg mL⁻¹ with a detection limit of 50 fg mL⁻¹. Meanwhile, the enzyme-free catalyst based immunosensor also showed acceptable selectivity, reproducibility and stability.

Keywords

Electrochemical immunosensor, Ag@Au nanorods, Fe₃O₄ nanospheres, Nanoelectrocatalyst

1. Introduction

A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Atkinson et al, 2001). Currently, there is a significant amount of interest in the development of analyte-specific immunoassays for the detection of low-abundance protein biomarkers. Compared with other immunoassay techniques, e.g. Fluorescence (Hu et al, 2016), surface-enhanced Raman scattering spectroscopy (Wang et al, 2013), enzyme-linked immunosorbent assay (Wang et al, 2016), electrochemiluminescence (Chen et al, 2015) and chemiluminescence (Zhang et al, 2014), electrochemical immunosensors have drawn much attention in recent decade because of their high sensitivity, miniaturizable instrumentation and possible analysis of difficult matrices without extensive pretreatment, therefore were widely applied in some trace amount of protein biomarkers detection (Wilson, 2005; Ma et al, 2015; Han et al, 2015; Zhao et al, 2016; Zhao et al, 2014; Yang et al, 2015; Wang et al, 2015a).

In order to meet the demand of a highly sensitive diagnostic assay for the fact that typical amount of protein biomarkers is of subnanomolar levels, signal amplification strategies achieved by coupling antibodies with different kinds of labels were frequently applied including electroactive nanoparticles (Ma et al, 2015), redox nanocomposites (Han et al, 2015), enzymes (Zhao et al, 2016; Zhao et al, 2014) and nanoelectrocatalysts (Yang et al, 2015; Wang et al, 2015a). Among them, the nonenzymatic electrocatalysts with superior catalytic activity were more attractive when compared with enzymes, because the problem of the loss of enzyme catalytic activity by environment changes could be avoided. So it was quite essential to develop promising enzyme mimic nanocatalysts for facilitating the redox reactions such as hydrogen peroxide reduction.

Fe₃O₄ nanoparticles (NPs) were often used in electrochemical immunosensors not only because of their large surface-to-volume ratio, strong adsorption ability and fast electron transfer, but also due to their intrinsic artificial enzyme mimetic activity (Wang and Tan, 2007; Yang et al, 2014). In particular, when loaded or coated with noble metal nanocatalyst like Pt and Au, the synergic catalytic effect toward the electrochemical reduction of H₂O₂ was dramatically increased (Wang et al, 2015b; Li et al, 2010). What's more, the separation of magnetic particles labeled biomacromolecules from solution with external magnetic fields is easy, time-saving and efficient. Recent research found Ag/Au alloy NPs provided greater catalytic activities for H₂O₂ reduction as compared to NPs of Au or Ag alone (Wang et al, 2012; Yang et al, 2013; Wang et al, 2014), especially in a shape-controlled manner (Yang et al, 2013). Motivating this work is the expectable catalytic activity of the resultant nanocomposite prepared by combining Fe₃O₄ NPs and Ag@Au nanorods (NRs).

Usually, coating with polymer shells is efficient to improve the stability and the further surface binding of the nanoparticles. Polydopamine (PDA) is a good choice for polymer coating due to its unique adhesive ability, good biocompatibility and easy bioconjugation (Ye et al, 2011; Liu et al, 2013a). PDA can be easily generated from the self-polymerization of dopamine and spontaneously deposited on virtually any surface to form a conformal layer. Furthermore, the strong adsorption ability and the existence of functional groups (i.e., catechol and amine) of PDA enhance the binding of various biomolecules (Lin et al, 2014; Lin et al, 2013).

Herein, we developed a nanohybrid of Ag@Au NRs and Fe₃O₄ nanospheres via the PDA coating, and then demonstrated a nonenzymatic electrochemical immunosensor

using the Ag@Au-Fe₃O₄ as an efficient nanocatalyst for H₂O₂ reduction. As shown in Scheme 1, the sensing mechanism is based on variation of reduction current of H₂O₂ catalyzed by Ag@Au-Fe₃O₄ which was introduced onto the electrode surface quantitatively related with the concentration of target protein due to the formation of sandwich type immunocomplex. The big surface area of Ag@Au-Fe₃O₄ also allows it to be an excellent carrier to load more antibodies for target protein binding. Meanwhile, carbon nanofibers-chitosan (CNFs-Chit) composite was modified to the sensing interface owing to the excellent electrical conductivity and functional surface for the covalent bonding of the capture antibodies. With human IgG as a model analyte, this sensor showed a wide linear range, low detection limit and some other encouraging performances.

Scheme 1. Schematic illustration of the preparation of the electrochemical immunosensor and the assay procedure.

2. Experimental

2.1. Materials and methods

Human IgG, mouse anti-human IgG antibody (anti-IgG) and bovine serum albumin (BSA) were obtained from Beijing Boishynthesis Biotechnology Co. Ltd. (Beijing, China). CNFs (Outer diameter 100 nm, length 20-200 μm) were purchased from Sigma. Glutaraldehyde (GA) and L-ascorbic acid (AA) were purchased from Kemiou Chemical Reagent Co. Ltd. (China). Cetyltrimethylammonium bromide (CTAB), chloroauric acid ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$), trisodium citrate, and silver nitrate were obtained from Shanghai Reagent Company (China). Sodium borohydride was obtained from Sinopharm Chemical Reagent Co. Ltd. (China). Tween-20 was obtained from MP Biomedicals. All other reagents were of analytical grade and used as received.

2.2. Preparation of CNFs-Chit nanocomposite

Firstly, 5.0 mg of chitosan was dissolved in 5.0 mL of acetic acid solution (2%) with magnetic stirring for about 2h. Then 5.0 mg of CNFs was dispersed into 5.0 mL chitosan solution with ultrasonication for 2h and a homogeneous CNFs-Chit nanocomposite suspension was prepared.

2.3. Preparation of Fe_3O_4 nanospheres, Ag@Au nanorods and $\text{Ag@Au-Fe}_3\text{O}_4$

Fe_3O_4 nanospheres were synthesized according to the literature reported previously with slight modification (Deng et al, 2005). Briefly, 0.625 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2.5 mmol) was dissolved in 20mL of ethylene glycol to form a clear solution, followed by adding 1.8 g NaAc and 0.5 g polyethylene glycol. The mixture was vigorously stirred for 30 min and then sealed in a 30 mL Teflon-lined autoclave. The autoclave was heated to 200 $^\circ\text{C}$ and kept for 10 h. The black products were separated and washed with ultrapure water and ethanol several times and then dried in vacuum at 40 $^\circ\text{C}$.

Ag@Au NRs were prepared by adding 2 mL of 0.1 M CTAB, 22 μL of 0.1 M AA, different amount of 0.01 M AgNO_3 and 50 μL of 0.25 M NaOH successively to 1 mL of the prepared Au NRs solution (Xiang et al, 2008) under vigorous stirring at 27 $^\circ\text{C}$.

To coat Fe_3O_4 cores with Ag@Au NRs, 10 mg of the above Fe_3O_4 were dispersed in 15 mL of Tris buffer solution (10 mM, pH = 8.5), followed by adding 5mL Ag@Au NRs and 10 mg of dopamine hydrochloride in the Fe_3O_4 suspension. After proceeding for 24 h at room temperature under stirring, the products were washed with ultrapure water several times and redispersed in water.

2.4. Preparation of $\text{Au@Ag-Fe}_3\text{O}_4\text{-Ab}$

The synthesized $\text{Ag@Au-Fe}_3\text{O}_4$ (150 μL , 2.5mg mL^{-1}) was dispersed into 10 ml of PBS buffer solution (0.1M, pH=7.4). The antibody (Ab) (35 μL , 1mg mL^{-1}) was added dropwise to the mixture under stirring. Then, the resulting solution was stirred for 24h

at 4 °C. Subsequently, the precipitate was separated by decantation with a magnet, thoroughly washed with PBS, and then redispersed in PBS and stored at 4 °C before use.

2.5. Fabrication of the immunosensor

Firstly, 6 μL of CNFs-Chit was drop-dried onto a glass carbon electrode (GCE). After dried at room temperature, the modified electrode incubated with 6 μL of 5% GA for 2.5 h, followed by washing with water. Then, 6 μL of 0.2 mg/mL anti-IgG (Ab_1) were applied to the CNFs-Chit/GCE surface, which was incubated at 4 °C overnight. Subsequently, excess antibodies were removed with PBST and PBS, respectively. Finally, a drop of 6 μL blocking solution (1% BSA) was dropped on the electrode surface and incubated for 30 min at 37 °C to block possible remaining active sites against nonspecific adsorption. After another washing with PBS and PBST, the immunosensor was obtained and stored at 4 °C in a dry environment prior to use.

2.6. Measurement

The immunosensor was firstly incubated at 37 °C for 1h with a certain concentration of IgG or serum samples. After a washing step, 6 μL of $\text{Ag@Au-Fe}_3\text{O}_4\text{-Ab}$ were further dropped onto the immunosensor and incubated for 30 min at 37 °C. After rinsing, the amperometric responses of the immunosensor were recorded with the working potential of -0.4 V . After the background current was stabilized, 20 mmol L^{-1} H_2O_2 unless other stated, were added into the buffer and the current change was recorded. The impedance analysis was performed over a range of frequencies from 0.1 Hz to 100 kHz using the amplitude of 10 mV at 0.2 V.

2.7. Apparatus

All electrochemical measurements were performed on a CHI660A electrochemical workstation (Shanghai CH Instrument Co. Ltd., China) using a three-electrode system with GCE or modified GCE as the working electrode, a saturated calomel electrode (SCE) and a platinum electrode as reference and counter electrode, respectively. The transmission electron microscopy (TEM) images were obtained from Tecnai G2 F20 S-TWIN (FEI, USA). Powder X-ray diffraction (XRD) patterns were performed using a Philip-X'Pert X-ray diffractometer with a $\text{Cu K}\alpha$ X-ray source. The scanning electron microscopy (SEM) image and the energy dispersive spectroscopy (EDS) spectra were performed on a JSM-6390A (JEOL).

3. Results and discussion

3.1. Characterization of Ag@Au-Fe₃O₄ nanohybrid

The SEM image of the as-prepared Fe₃O₄ nanoparticles was investigated (Fig. 1A). It exhibited homogeneous sphere-shaped nanoparticles with rough surface morphology and narrow size distribution (Fig. S1A). TEM was utilized to investigate the morphology of the Ag@Au NRs and PDA coating enhanced Ag@Au assembly on the Fe₃O₄ nanospheres (Zheng et al, 2014). TEM image (the inset of Fig. 1B) of the Ag@Au nanostructures showed a smooth surface of Ag shell on the Au NRs. For a bimetal core-shell NRs, the metal with higher atomic number usually shows dark image and the metal with lower atomic number shows gray/light image (Jayabal and Ramaraj, 2014). The dark and gray/light contrast difference in the TEM image observed for the Ag@Au NRs indicates that a uniform thickness of the Ag layer was coated on the surface of the Au NRs in the core/shell structure. From the TEM image of the nanohybrid (Fig. 1B and Fig. 1C), well-defined sunflower pattern nanostructure wrapped by PDA coating layer was observed, which indicated the successful assembly of Ag@Au NPs on the surface of Fe₃O₄ nanospheres. Ag@Au nanoparticles here (Fig. 1C) showed a small length to diameter ratio compared with it of Ag@Au NRs showed in Fig. 1B. This is because the silver layer deposited on gold nanorods was prepared with different amount of AgNO₃. As shown in Fig. 1D, the suspension of Ag@Au-Fe₃O₄ nanohybrids is homogeneous. The Ag@Au-Fe₃O₄ nanohybrid was attracted immediately toward the magnet once an external magnetic field is applied, leaving the bulk solution clear and transparent. Therefore, the nanohybrid can be separated and cleaned conveniently owing to the advantage of the inherent magnetic property of the Fe₃O₄ NPs.

The energy dispersive X-ray spectroscopy measurement of the Ag@Au-Fe₃O₄ revealed the existence of C, N, O, Fe, Ag and Au elements (Fig. S1B), confirming the successful formation of Ag@Au-Fe₃O₄ nanocomposite via the one-pot in-situ PDA coating. We also applied XRD to get insight into the crystal formation of Au and Ag nanocrystals in the composite. XRD patterns of Fe₃O₄, Ag@Au, and Ag@Au-Fe₃O₄ were presented in Fig. 1E. The XRD profiles of Fe₃O₄ nanospheres revealed the presence of diffraction peaks at (111), (200), (220), (311), (331), (400), (440) and (511) related to the Fe₃O₄ phase. The XRD pattern of Ag@Au also exhibited peaks at (111), (200), (220), (311), which belonged to the crystal plane of bimetallic Ag@Au (Sun et

al, 2015). Two sets of peaks from the Ag@Au-Fe₃O₄ nanohybrid matched well with the peaks of Ag@Au nanorods and Fe₃O₄ nanospheres, respectively, confirming the composition of the nanohybrid.

Fig.1. Characterization of the related nanocomposite. (A) SEM image of Fe₃O₄ NPs. (B) TEM image of Ag@Au-Fe₃O₄ nanohybrids, the inset shows a TEM image of Ag@Au NRs. (C) TEM image to highlight one Ag@Au-Fe₃O₄ nanohybrid sunflower. (D) Photos of Ag@Au-Fe₃O₄ with external magnetic effect. (E) XRD of Fe₃O₄ NPs, Ag@Au NRs and Ag@Au-Fe₃O₄ nanohybrids.

Electrochemical characteristic of Ag@Au-Fe₃O₄ was investigated by drop coating the suspension of the nanomaterial on a clean GCE. The anodic peak at ca. 0.1 V and cathodic peak at -0.14 V for the oxidation of silver and corresponding reduction process (Ma et al, 2014) appeared on the cyclic voltamogram (Fig. S2A) of Ag@Au NRs/GCE and Ag@Au-Fe₃O₄/GCE and disappeared on the cyclic voltamogram of Fe₃O₄/GCE, which was also an evidence for the formation of Ag@Au-Fe₃O₄ nanocomposite. Furthermore, both the anodic and cathodic peak currents at Ag@Au-Fe₃O₄/GCE showed a dramatic increase when compared with the peak current obtained at Ag@Au NRs/GCE, reflecting that more Ag@Au NRs were attached on the electrode surface by loading on the Fe₃O₄ nanospheres. Moreover, cyclic voltamograms of Fe₃O₄/GCE, Ag@Au NRs/GCE and Ag@Au-Fe₃O₄/GCE in 0.1M KCl solution containing 5 mM ferricyanide at different scan rates were recorded to calculate the effective surface area of the three modified electrodes according to

the relationship of peak currents and scan rates (Fig. S2B) for a reversible couple given by Randles-Sevcik equation (Lee et al, 2015; Liu et al, 2013b), $i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} \nu^{1/2} c$, where i_p (A) is the anodic peak current, n is the number of electron transfers, A (cm^2) is the electrode surface area, c (mol cm^{-3}) is the concentration of $\text{K}_3[\text{Fe}(\text{CN})_6]$, D ($\text{cm}^2 \text{s}^{-1}$) is the diffusion coefficient, and ν (V s^{-1}) is the scan rate. From the results, it was calculated that the effective working area of $\text{Fe}_3\text{O}_4/\text{GCE}$, $\text{Ag@Au NRs}/\text{GCE}$ and $\text{Ag@Au-Fe}_3\text{O}_4/\text{GCE}$ is 0.047, 0.059 and 0.063 cm^2 , respectively. These results indicated that $\text{Ag@Au-Fe}_3\text{O}_4/\text{GCE}$ possessed the largest surface area for electronic transfer (Lee et al, 2015).

3.2. Electrocatalytic characteristic of $\text{Ag@Au-Fe}_3\text{O}_4$ toward hydrogen peroxide reduction

To compare the electrocatalytic characteristic of the nanomaterial modified electrode toward hydrogen peroxide reduction, CVs of (A) $\text{Fe}_3\text{O}_4/\text{GCE}$, (B) $\text{Au NRs}/\text{GCE}$, (C) $\text{Ag@Au NRs}/\text{GCE}$ and (D) $\text{Ag@Au-Fe}_3\text{O}_4/\text{GCE}$ in N_2 -saturated 0.1 M PBS (pH 7.4) in the absence (a) and presence (b) of 5.0 mM H_2O_2 were recorded. As shown in Fig. 2A, the typical CV of $\text{Fe}_3\text{O}_4/\text{GCE}$ in the absence of H_2O_2 showed a small background current response which is similar with the response of bare GCE (Fig. S2C). In the presence of H_2O_2 , there is a great increase in the cathodic current from the potential around -0.2 V. This is caused by the electrocatalytic reduction of H_2O_2 by Fe_3O_4 NPs (Wei and Wang, 2008). When compared with the reductive current of bare GCE (Fig. S2C), the current increase of $\text{Fe}_3\text{O}_4/\text{GCE}$ for H_2O_2 reduction is obvious. This is in accordance with the peroxidase enzyme mimic feature of Fe_3O_4 nanospheres (Zuo et al, 2009; Shamsipur et al, 2015). For the $\text{Ag@Au NRs}/\text{GCE}$, after the addition of 5 mM H_2O_2 in 0.1 M PBS (pH 7.4), an increase of reduction current with a strong peak intensity at -0.54 V was observed, indicating that Ag@Au NRs also exhibited a notable catalytic activity for the reduction of H_2O_2 (Fig. 2C). The response of $\text{Au NRs}/\text{GCE}$ was recorded to compare (Fig. 2B). The reductive current at the $\text{Au NRs}/\text{GCE}$ is apparently weaker than that of at the $\text{Ag@Au NRs}/\text{GCE}$. This was also observed in the previous studies (Yang et al, 2013), which demonstrated that the Ag/Au bimetallic NPs exhibited more effective performance on H_2O_2 reduction than their mono-component counterparts (Shamsipur et al, 2015). As is depicted in Fig. 2D, the $\text{Ag@Au-Fe}_3\text{O}_4/\text{GCE}$ displayed the largest reduction current among the four kinds of modified electrodes, indicating a strong catalytic effect of the modified nanocatalysts. The excellent electrocatalytic activity of the

Ag@Au-Fe₃O₄/GCE should be benefited by the synergy effect of Ag@Au NRs and the magnetic nanospheres. Normally, a larger surface area will lead to a larger electrochemically active surface area (Yin et al, 2015). The relatively big surface area of Ag@Au-Fe₃O₄ nanocomposite increased the catalytic sites of Ag@Au NRs and accelerated the electron transfer rate of Fe₃O₄ NPs.

The effect of Ag shell thickness on the performance of the Ag@Au-Fe₃O₄/GCE was examined. Firstly, the thickness of Ag shells was controlled by coating of the same amount of the Au NRs with different volumes of 0.01 M AgNO₃ (Xiang et al, 2008). Then, the Ag@Au NRs were coated on the Fe₃O₄ nanobead to form Ag@Au-Fe₃O₄. The amperometric responses Ag@Au-Fe₃O₄/GCE prepared by Ag@Au NRs with different silver thickness toward the reduction of 5 mM H₂O₂ were investigated (Fig. S3). The responses of the Ag@Au-Fe₃O₄ with different Ag shell thickness were slightly different. Ag@Au NRs prepared with 400 μ L AgNO₃ was applied in the subsequent experiments to obtain a relatively sensitive and stable signal.

Fig.2. CVs of (A) Fe₃O₄/GCE, (B) Au NRs/GCE, (C) Ag@Au NRs/GCE and (D) Ag@Au-Fe₃O₄/GCE in N₂-saturated 0.1 M PBS (pH 7.4) in the absence (a) and presence (b) of 5.0 mM H₂O₂ at a scan rate of 50 mV s⁻¹.

3.3. Electrochemical characterization of the immunosensor

The electrochemical impedance spectroscopy (EIS) and cyclic voltamograms of different electrodes were recorded in a solution containing 0.1 M KCl and 5 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ to monitor the impedance changes of the interface during the electrode preparation process (Fig. S3) (Wang et al, 2014). The bare GCE exhibited a small semicircle diameter on EIS and a redox peak on CV, suggesting a reversible diffusion-controlled electrochemical process. After CNFs-Chit was modified on the surface of the GCE, the semicircle part on Nyquist plot was hardly to be seen, showing a neglectable interface impedance which is attributed to the excellent electrical conductivity of CNFs-Chit. At the same time, the peak current on CV increased due to increasing effective surface area in the presence of the nanocomposite. Subsequently, the resistance values and peak current changed gradually with the incubation of anti-IgG antibodies (Ab_1), BSA and IgG, indicating that the protein film was covered on the electrode successfully and blocked electron transfer between the base solution and electrode (Liu et al, 2015).

Fig.3. Amperometric response of the immunosensor for detecting 1 ng mL^{-1} IgG with different label at -0.4 V vs. SCE toward successive addition of $5\text{ mM H}_2\text{O}_2$ in N_2 -saturated PBS; (a) $\text{Fe}_3\text{O}_4\text{-Ab}_2$, (b) Ag@Au-Ab_2 , and (c) $\text{Ag@Au-Fe}_3\text{O}_4\text{-Ab}_2$ and their corresponding schematic diagram. Inset is the magnification of curve a.

The primary determinant of the sensitivity of the immunosensor based on Ag@Au-Fe₃O₄ is the amperometric response of the sensor using the enzyme mimic nanoelectrocatalyst as labels. To further demonstrate the immunosensing application of the Ag@Au-Fe₃O₄, herein, the chronoamperometry plots of the immunosensors prepared with different bioconjugate of Fe₃O₄-Ab₂, Ag@Au-Ab₂, and Ag@Au-Fe₃O₄-Ab₂ were investigated in pH 7.4 PBS. As showed in Fig. 3, for the immunosensor using Fe₃O₄-Ab₂, a relatively-small current response with the addition of H₂O₂ was yielded (curve a). For the immunosensor using Ag@Au-Ab₂, a much larger current response (curve b) was displayed upon addition of H₂O₂. The immunosensor with Ag@Au-Fe₃O₄-Ab₂ showed a drastically improved current response (curve c). The facts indicated that the synergistic effect between Fe₃O₄ nanospheres and Ag@Au NRs enhanced the catalysis toward H₂O₂ reduction. Therefore, Ag@Au-Fe₃O₄ nanohybrid is an excellent label in the fabrication of nonenzymatic electrochemical immunosensor.

3.4. Optimization of experimental conditions

The electrochemical performance of the immunosensor is influenced by parameters including concentration of H₂O₂, solution acidity and incubation time. The catalytic substrate concentration of H₂O₂ had a crucial impact on the current response of the immunosensor. The current response of the immunosensor increased with increasing concentration of H₂O₂ from 2.5 to 30 mM and tended to balance from 20 to 30 mM (Fig. S5A). 20 mM H₂O₂ was chosen as the optimum concentration (Gao et al, 2015). The pH of the solution has a great effect on the electrochemical behavior of the immunosensor because the activity of the immobilized protein may be influenced by the acidity of the solution (Gao et al, 2014; Guo et al, 2015; Katz and Willner, 2005). In order to optimize the pH, a series of PBS buffer with the pH from 4.5 to 9.0 were prepared and the pH of the PBS was investigated with same concentration of Ag@Au-Fe₃O₄ modified on the surface of GCE. As shown in, the current signal increased with the increase of pH from 4.5 to 7.4, and then decreased with the further pH increase from 7.4 to 9.0 (Fig. S5B). The largest electrochemical signal was achieved at pH value of 7.4. Therefore, PBS at pH 7.4 was chosen as the electrolyte for all other electrochemical tests. Incubation temperature is another important parameter in the performance of the immunosensor. The effect of temperature on the amperometric response of the sensor was investigated at range from 25 to 45°C. The amperometric current increased as the temperature increased until they reach a

maximum value at 37°C, which indicated that the formation of antibody-antigen immunocomplex was gradually promoted, whereas they decreased when temperature is over 40°C. It may be due to the irreversible behavior (denaturation of proteins) involved in the process which is caused by the high temperature (Qu et al, 2007). Thus, 37°C is the suitable incubation temperature.

Fig.4. (A) Amperometric response of the immunosensor toward addition of 20 mM H_2O_2 for detecting of different concentration of IgG from 0.0001, 0.001, 0.01, 0.1, 0.5, 1, 10, 100, 500, 1000, 5000 and 10000 ng mL^{-1} at -0.4 V vs. SCE. (B) Calibration curve of the immunosensor toward different concentrations of IgG ($n=5$).

3.5. Performance of the immunosensor

To evaluate the analytical performance of the Ag@Au- Fe_3O_4 based immunosensor, a series of IgG solution was measured under the optimized conditions described above. The response of the immunosensor toward IgG increased with increasing the concentration of the analyte (Fig. 4). The calibration plot showed a good linear relationship between the catalytic current change and the logarithm values of the analyte concentration in the broad range from 0.1 pg mL^{-1} to 5 $\mu\text{g mL}^{-1}$. The linear regression equation was expressed as $I (\mu\text{A}) = 23.04 + 28.22 \log C (\text{pg mL}^{-1})$ with a correlation coefficient of 0.994. The limit of detection at a signal-to-noise ratio of 3 was estimated to be 0.05 pg mL^{-1} . The analytical performance of this immunosensor was superior to several other immunoassays (Table 1). The excellent performance can be attributed to several factors. Firstly, the modification of CNFs-Chit nanocomposite promoted the electron transfer. Meanwhile, Ag@Au- Fe_3O_4 increased the probability

of Ab₂-antigen interactions when compared with Ag@Au NRs and Fe₃O₄ NPs as shown in Fig.3. Most importantly, the high electroactivity of Ag@Au-Fe₃O₄ nanoelectrocatalyst greatly amplified the signal. Moreover, this Ag@Au-Fe₃O₄ nanoelectrocatalyst immunosensor can be used repetitively for at least twenty times which may reduce the sensor cost due to its H₂O₂ reduction based signal resource which is different with our previous work (Ma et al, 2015; Zhang et al, 2015).

Table 1. Comparison of the analytical performance of different immunoassays for detection of IgG.

Linear range ng mL ⁻¹	Detection limit pg mL ⁻¹	Stability	Sensitivity μA mL ng ⁻¹	References*
—	5.6×10 ⁴	—	—	(Ertürk et al, 2011) ^a
1×10 ⁻⁴ –0.5	0.05	2 weeks	—	(Chen et al, 2015) ^b
5×10 ⁻³ –50	1.5	—	—	(Hu et al, 2016) ^c
0.5–5×10 ⁷	0.5	—	—	(Cho et al, 2015) ^d
5–100, 100–3×10 ³	3×10 ³	—	—	(Lee et al, 2011)
0.82–90	250	90% after 20 days	—	(Zhang et al, 2008)
0.1–1×10 ⁵	50	91.5% after 1 month	4.53×10 ⁻⁶	(Zhao et al, 2016)
0.5–125	20	92.9% after 14 days	5.8**	(Tabrizi et al, 2016)
0.01–100	1	96.6% after 15 days	2.21	(Zhang et al, 2016)
0.01–200	4	93.3% after 2 weeks	2.97	(Cao et al, 2013)
1×10 ⁻⁴ –5000	0.05	95.7% after 3 weeks	28.22×10 ³	This work

*Measurement protocol: (a) surface plasmon resonance; (b) enzyme linked immunosorbent assay; (c) fluorescence; (d) photoluminescent, the unit of the data is pM; the others are all electrochemical immunosensors.

**the unit of the data is Ω mL ng⁻¹.

3.6. Selectivity, reproducibility and stability of the immunosensor

The performance of the immunosensor toward IgG was investigated in the presence of vitamin C (AA), glucose (Glu), hemoglobin (Hb), human albumin (HSA) and bovine albumin (BSA). From Fig. 5, the amperometric response of the immunosensor toward 1.0 ng mL⁻¹ interference and the mixture of the interference with equivalent amount of IgG were measured, individually. Much higher current was observed for IgG than those of interfering substances, indicating that there is no significant interference from the tested non-specific species to the immunosensor in our experiments. Meanwhile, the current change owing to the mixture containing IgG and the interfering substances was less than 3.9% of that owing to IgG alone. All these observations indicate that the immunosensor exhibits an acceptable selectivity for the determination of IgG.

To evaluate the reproducibility of the immunosensor, the immunosensors prepared with five individual electrodes were applied to the detection of 1 ng mL^{-1} IgG. The coefficient of variation for the five immunosensors was 4.1%, indicating good reproducibility of the immunosensor (Fig. S6A). In addition, the stability of the immunosensor was studied. It was stored at 4°C in air after the first use, and then the signal was measured weekly. The initial response only decreased 4.3% after a storage period of three weeks (Fig. S6B). This demonstrated the satisfactory stability of the immunosensor.

Fig.5. Amperometric response of the immunosensor to blank, 1 ng mL^{-1} AA, IgG, Hb, BSA, Glu, HSA, 1 ng mL^{-1} IgG+ 100 ng mL^{-1} Hb, 1 ng mL^{-1} IgG+ 100 ng mL^{-1} BSA, 1 ng mL^{-1} IgG + 100 ng mL^{-1} HSA, 1 ng mL^{-1} IgG + 100 ng mL^{-1} Glu, and 1 ng mL^{-1} IgG + 100 ng mL^{-1} AA.

3.7. Real sample analysis

In order to evaluate the feasibility of the immunosensor for possible application to practical analysis, the recoveries of different concentrations of IgG in human serum were detected. Different levels of IgG were spiked into the serum samples considering the linear range of the immunosensor. The results showed that the recovery was in the range of 94.0 to 106.0% and RSD was less than 2.7% (Table S2), indicating good accuracy of the proposed method for sample detection.

4. Conclusion

In summary, we introduced a novel nonenzymatic electrochemical immunosensor. This immunosensor relies on the excellent catalytic reduction ability of the elaborately-designed $\text{Ag@Au-Fe}_3\text{O}_4$ nanoelectrocatalyst toward H_2O_2 , which was

evidenced by the comparison with the catalytic ability of Ag@Au NRs and Fe₃O₄ nanospheres. We presented the excellent analytical performance of the obtained immunosensor using IgG as the model analyte. The sensor exhibited broad linear range, low detection limit, high selectivity, acceptable reproducibility and good stability. Therefore, it is possible to foresee that the immunosensing platform might be adapted to measure the other protein biomarkers.

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