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Electrochemical immunosensor of tumor necrosis factor α based on alkaline phosphatase functionalized nanospheres

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ABSTRACT

A novel immunosensor for sensitive detection of tumor necrosis factor α was reported. First of all, gold nanoparticles were uniformly assembled on the surface of poly (styrene-acrylic acid) nanospheres, which was used as the matrix to conjugate alkaline phosphatase (ALP). And then, the obtained composite was used as multi-enzyme functionalized label for immunoassay. Biocompatible polyaniline doped with poly (acrylic acid) was electro-polymerized at the glass carbon electrode to construct the matrix for the immobilization of antibody TNF-α. After the sandwich immunoreaction, the labeled ALP was used to hydrolyze α-naphthyl phosphate to produce the electroactive α-naphthol, which could be amperometrically detected. The results showed that the electrochemical signals were proportional to the logarithm of the antigen concentration in the range of 0.02–200.00 ng/mL with the detection limit of 0.01 ng/mL. The developed immunoassay showed high sensitivity, acceptable stability and reproducibility, which might have potentially broad applications in protein diagnostics and bioassay.

1. Introduction

In clinic diagnoses, many diseases have the corresponding expressional signal via the biomarker. Tumor necrosis factor α (TNF-α) is one of the biomarkers, which involves the wide range of pathological and physiological process. For example, elevated TNF-α concentration in serum has been associated with a broad series of pathological states, such as neonatal listeriosis, severe meningococcemia, HIV infection, systemic erythema nodosum leprosum, endotoxic shock, graft rejection, and rheumatoid arthritis (DeKossodo et al., 1995). Generally, TNF-α is at low level in biological samples. Therefore, the development of sensitive methods for the detection of trace biomarkers is very important for the understanding of tumor biological process, inherent mechanism, discovering drugs, and it has a therapeutic potential for the treatment of diseases.

Sandwich-type immunoassay is an effective bioassay due to the high specificity and sensitivity (Knopp, 2006). Several antibodies functionalized with various labels, including radioisotopes (Higashi et al., 2005), enzymes (Zhang and Heller, 2005), fluorescence compounds (Chan et al., 2004), and metal compounds (Fan et al., 2005) were used in the immunoassay. Alkaline phosphatase (ALP) is a commonly used enzyme that can hydrolyze a variety of phosphate esters to orthophosphate under alkaline conditions of pH 8–10. Lin (Lee et al., 2008) and Chailapakul (Preechaworapun et al., 2008) have successfully used ALP labeled object biomolecules for electrochemical determination of IgG and DNA. At the same time, it is important to amplify the electrochemical signal to obtain low detection limits and high sensitivities (Tang et al., 1991; Fu et al., 2006; Long et al., 2005). Typically, Yang presented a signal amplification method using p-aminophenol redox cycling by hydrazine based on ALP labeled secondary antibody, and consequently the sensitivity and linear range were evidently optimized (Das et al., 2007). However, the immunosensor regarding the labeled multi-ALP has not been reported.

Gold nanoparticles (GNPs) have received increasing attention, due to their inherent advantages, such as good biocompatibility, stability, easy preparation, and so on, and have been widely applied in bioelectrochemistry (Georganopoulou et al., 2005; Katz and Willner, 2004; Zhang et al., 2002; Daniel and Astruc, 2004; Gittins et al., 2000; Mani et al., 2009). In electrochemical immunoassay, GNPs and their hybrid materials have been used as the label matrix to conjugate enzyme (Cui et al., 2008a,b; Tang et al., 2008; Tang and Ren, 2008). These nanoparticle-based amplification platforms can dramatically enhance the intensity of the electrochemical signal and lead to ultrasensitive bioassays (Wang, 2005; Daniel and Astruc, 2004).

Herein, we report a novel strategy for the detection of TNF-α. A film of polyaniline doped with poly (acrylic acid) was electropolymerized by cyclic voltammetry to introduce carboxyl groups for conjugating TNF-α antibody. The following sandwich-type

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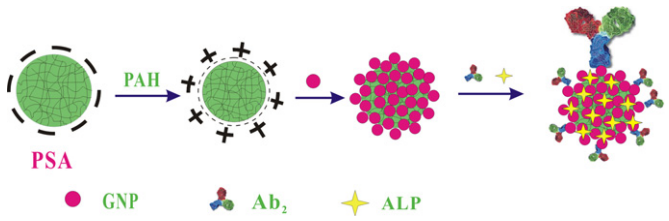

Fig. 1. Assembly procedure of ALP-Ab_2-GNPs/PSA bioconjugates.

immunoassay was conducted using the label of multi-ALP functionalized GNPs/poly (styrene-co-acrylic acid) (GNPs/PSA) based on gold nanoparticles, and ALP can hydrolyze α-naphthyl phosphate (p-NPP) to produce the electroactive α-naphthol (p-NP). The quantity of antigen can be determined by the electrochemical signals of p-NP. To the best of our knowledge, this is a new immunoassay with multi-ALP based labels.

2. Experimental

2.1. Reagents and apparatus

Tumor necrosis factor α (TNF-α) (antigen), TNF-α antibody (antibody), human IgG antigen (HIgG), carcinoembryonic antigen (CEA), alkaline phosphatase (ALP, 14 U/mg), α-napthyl phosphate, lyophilized bovine serum albumin (BSA, 99%), Tween-20, poly (acrylic acid) (PAA, MW = 100 000), poly (allylamine hydrochloride) (PAH, MW = 15 000), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma. Styrene and aniline were purchased from Beijing Chemical Reagent Factory (Beijing, China) and were purified before used. $K_2S_2O_8$ was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). All other reagents were of analytical reagent grade and used without further purification. Doubly distilled water was used throughout the experiments.

Electrochemical immunoassay was performed on a CHI 660B electrochemical analyzer (Chenhua, Shanghai, China) with a conventional three electrode system comprised of platinum wire as the auxiliary electrode, saturated calomel electrode (SCE) as the reference electrode and glass carbon electrode (GCE) as the working electrode. The electrochemical impedance spectroscopy analyses were performed in the solution of 0.10 M KCl containing 2.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ under the conditions of the frequency range $(0.1–1.0) \times 10^5$ Hz, the formal potential (0.20 V versus SCE) of the redox couple and perturbation potential of 5 mV. The morphology of the composite was observed by high-resolution transmission electron microscope (HRTEM, JEM-2100) with an accelerating voltage of 200 kV. The sample for TEM analysis was prepared by the addition of the solution with particles onto a standard holey carbon coated copper grid. The grid was then dried under ambient conditions. UV–vis absorption spectra were recorded on a UV-2401PC spectrometer.

2.2. Preparation of ALP-Ab_2-GNPs/PSA bioconjugates

The fabrication process of ALP-Ab_2-GNPs/PSA is shown in Fig. 1. The PSA particles were prepared by emulsifier-free emulsion polymerization method (Polpanich et al., 2005; Xiao et al., 2004) with some alteration. A mixture of styrene (5.0 g) and acrylic acid (1.0 g) was dissolved in 150 mL of deionized water in a 250 mL three-necked round-bottom flask which was equipped with a condenser, a nitrogen inlet, and a thermometer. After purging with nitrogen for about 1 h to remove oxygen under stirring, polymerization was initiated by adding $K_2S_2O_8$ (0.10 g, 5 mL) into the mixture at 70 °C. The reaction continued for 8 h at 70 °C under stirring. The resulting PSA latex suspensions were purified by repetitive centrifugation and redispersion in the same volume aqueous solution. Then the purified polymer spheres were functionalized with PAH. Firstly, 0.1 mL polymer spheres were dispersed into 5 mL aqueous solution of 0.20% PAH and 20 mM NaCl, and the resulting dispersion was ultrasonicated for 30 min to give a homogeneous suspension. Residual PAH was removed by high-speed centrifugation and the complex was rinsed with water for five times. The hybrid was dispersed in gold colloid solution which was prepared according to the literature (Enustun and Turkevich, 1963) and stirred for 30 min. Then the light-purple GNPs/PSA composite was obtained. The composite was further washed with water for three times and redispersed in 5 mL aqueous solution.

At room temperature, 4 μL of 5.0 mg/mL ALP and 20 μL 0.5 mg/mL TNF-α was added into 1.0 mL of the prepared GNPs/PSA solution containing 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide (Cui et al., 2008a; Hayat, 1989). The mixture was gently mixed for 6 h, blocked by 50 μL of 1% BSA solution for 20 min at room temperature, and centrifuged at 10 000 rpm for 8 min at 4 °C. After centrifugation, the composite was washed with distilled water and redispersed in 3 mL of Tris–HCl (pH 9.0) as the experimental solution. The ALP functionalized GNPs (ALP-GNPs-Ab_2) were fabricated as the similar processes.

2.3. Fabrication of immunosensor

A polyaniline/poly (acrylic acid) (PANA) composite layer was generated at glass carbon electrode by the electro-polymerization of 0.2 M aniline containing 15 mg/mL PAA in the electrolyte solution of 0.1 M H_2SO_4 and 0.5 M Na_2SO_4 (pH 1.8) (Raitman et al., 2002). The polymerization was performed by one cyclic scan between −0.1 and +1.1 V at 100 mV s^{-1}. The resulting film was washed with the solution of 0.1 M H_2SO_4 and 0.5 M Na_2SO_4 to remove the residual monomer at the surface.

The covalent coupling of Ab_1 at the PANA surface was performed by subsequently coating with 20 μL of 5 mM NHS/EDC solution for 2 h at room temperature, and then the supernatant was removed. 20 μL of 25 μg/mL TNF-α was used to cover the electrode. After incubation for 3 h, the solution was removed, and the electrode was blocked with 20 μL of 2% BSA solution containing 0.05% Tween-20 for 40 min at room temperature. After the solution was washed off, the modified electrodes were washed for five times with the solution of 10 mM Tris–HCl (pH 9.0) and stored at 4 °C.

2.4. Assay procedure

The immunoassay protocol is shown in Fig. 2. The immunosensor was firstly incubated with the target TNF-α antigen for 60 min at room temperature. After the sensor was washed with PBS, it was put into the solution of ALP-Ab_2-GNPs/PSA for 60 min. The ALP conjugated nanospheres were brought onto the electrode surface by the binding reaction between Ab_2 and Ag. After the immunosensor was washed thoroughly with water to remove non-specifically bounded conjugates, the modified electrode was incubated in 3 mM p-NPP solution (0.1 M Tris–HCl pH 9.0) for 10 min, then a differential pulse voltammetry (DPV) scan from 0 to 0.5 V was recorded. The stock substrate solution of p-NPP was prepared daily in Tris–HCl buffer solution.

3. Results and discussion

3.1. Polymerization and characterization

Curve a of Fig. S1A showed the first cyclic voltammogram (CV) in 0.2 M aniline with potential from −0.1 to 1.1 V. Curve b of Fig. S1A

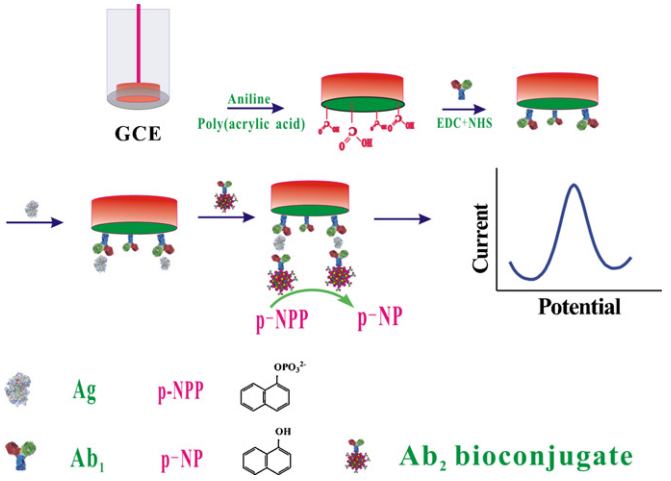

Fig. 2. The analytical procedure of PANA-based immunosensor using ALP-Ab_2-GNPs/PSA bioconjugates as labels.

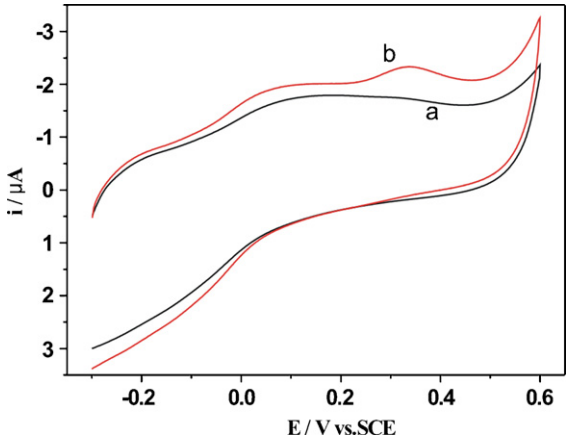

Fig. 3. CVs of immunosensors immunoreacted with (a) Ab_2-GNPs/PSA and (b) ALP-Ab_2-GNPs/PSA in 0.1 M Tris buffer solution (pH 9.0) containing 3 mM p-NPP. The scan rate was 100 mV s^{-1}.

is the CV curve in aniline (0.2 M) and PAA (15 mg/mL). An irreversible oxidation peak at ~0.70 V was observed in the first cycle, which was believed to be the oxidation of aniline to free radicals at the GCE. It was obvious that the current of the oxidation peak greatly decreased in the presence of PAA. After the electropolymerization, the electrode exhibited redox processes in blank solution as shown in Fig. S1B. A couple of peaks were localized at 0.41 and 0.46 V. When incorporating the counter-ions of PAA into polyaniline during the oxidative polymerization of aniline, the redox peak was smaller than that of the polymerization of aniline. The results revealed that less aniline was polymerized at the electrode in the presence of PAA than those in the absence of PAA (Raitman et al., 2002).

The technique of electron impedance spectroscopy (EIS) was effective to monitor the surface features, which could be used as a parameter to understand the chemical transformations and processes associated with the conductive surface (Bard and Faulkner, 1980). The EIS was composed of a semicircle portion and a linear portion. The semicircle portion made from higher frequencies corresponded to the electron-transfer limited process, and the linear part at lower frequencies corresponded to the diffusion process. The semicircle diameter was the characteristics of the electron-transfer resistance (R_{et}). Fig. S1C shows the nyquist plots of EIS for the bare GCE, PANA/GCE, and Ab_1/PANA/GCE, respectively. The probe of the $[Fe(CN)_6]^{3+/4+}$ showed zero R_{et} (curve a) at bare GCE. However, the PANA GCE showed a resistance of about 300 Ω (curve b), which suggested that the hybrid layer of negatively charged carboxylic groups and polyaniline limited the diffusion of ferri/ferro cyanide to the electrode surface. After the Ab_1 molecules were combined covalently on the PANA electrode, the inter-facial resistance increased. The result indicated that the protein layer on the electrode generated a barrier for electron-transfer, which was similar to the previous report (Pei et al., 2001). On the other hand, the phenomena also showed that the hybrid polymer-based surface was an effective matrix to immobilize antibody.

The GNPs prepared by the reduction of citrate have negative charges, which can adhere on surface with positive charges by electrostatic attraction. Fig. S2A is the TEM image of the fabricated hybrid. The GNPs can be uniformly distributed on the spheres. Meanwhile, UV–vis absorption spectra of the three systems are shown in Fig. S2B. The characteristic peak of citrate-stabilized GNPs appeared at 517 nm. Compared with the spectrum of pure colloidal PSA spheres (curve a), a new absorption band centered at 542 nm was observed in the spectrum of the GNPs/PSA (curve c), which indicated the enrichment of GNPs on the PSA surface (Guarize et al., 2005; Thomas et al., 2004).

3.2. The immunoassay using the labels of ALP-Ab_2-GNPs/PSA bioconjugates

ALP can catalyze the hydrolysis reaction of p-NPP to produce the electroactive species of p-NP. Fig. 3 shows the typical CV response for the immunosensor. After incubated with ALP-Ab_2-GNPs/PSA label, an obvious oxidation peak was observed at 0.3 V in 0.1 M pH 9.0 Tris–HCl buffer solution containing 3 mM p-NPP (Fig. 3b). However, no amperometric response was observed after incubation with Ab_2-GNPs/PSA label (Fig. 3a), which suggested that electroactive p-NP was generated only in the presence of ALP.

DPV is an electrochemical technique which can show higher sensitivity for determination. Fig. 4A shows the typical DPV responses for the detection of antigen using the ALP-Ab2-GNPs/PSA bioconjugates as the label. In a controlled experiment, the sensor was performed through the full procedure without exposure to antigen, and the final electrode still behaved an unconspicuous peak in the solution of p-NPP, which might be caused by the physical adsorption of the ALP functionalized nanoshperes at the GCE and direct hydrolysis to produce the electroactive p-NP. However, the DPV signals were obviously enhanced in the presence of antigen, and a linear relationship between the background-subtracted peak current versus the logarithm of the concentration of Ag (Fig. 4C, curve a) was observed. The linear response was over the range from 0.02 to 200.00 ng/mL and the detection limit was about 0.01 ng/mL which was similar as the reported values using a QD-based and poly (guanine)-based electrochemical immunoassay (Liu et al., 2006; Wang et al., 2006).

On the other hand, ALP functionalized GNPs (ALP-Ab2-GNPs) were also prepared and used as the label in the same system. Similar analytical steps as summarized for the ALP-Ab_2-GNPs/PSA bioconjugates were followed in these processes. Fig. 4B shows the typical DPV signals. The peaks intensity was proportional to the logarithm of the concentration of the antigen (Fig. 4C, curve b). The linear relationship was observed from 0.15 to 100.00 ng/mL with the detection limit of 0.05 ng/mL. As shown in Fig. 4C, the signal intensity from the label of ALP-Ab_2-GNPs/PSA was greatly enhanced than that of only using ALP-Ab_2-GNPs as label. And the detection limit of using ALP-Ab_2-GNPs/PSA as label was about five times lower than that of using the Ab_2-GNPs-ALP conjugates. The reason might be the fact that more ALP molecules were conjugated on the GNPs/PSA nanospheres. When one antibody molecule on the surface of ALP-GNPs/PSA reacted with the corresponding anti-

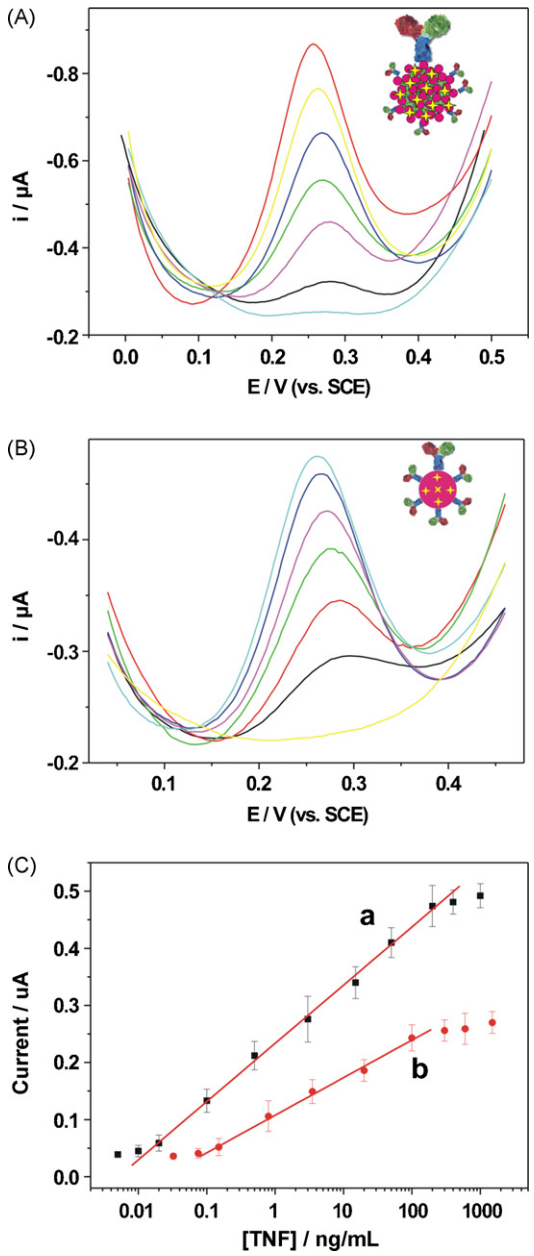

Fig. 4. Typical DPVs using the labels of (A) ALP-Ab_2-GNPs/PSA bioconjugates with increasing TNF-α concentration from bottom to top (0.0, 0.1, 0.5, 3.0, 15, 50 and 200 ng/mL TNF-α, respectively), (B) ALP-GNP-Ab_2 with increasing TNF-α concentration from bottom to top (0.0, 0.8, 3.5, 20, 100, 1500 and 1700 ng/mL TNF-α, respectively) (electrolyte: 0.1 M Tris–HCl pH 9.0 solution containing 3 mM p-NPP and incubated for 10 min before scanning; DPV: $E_{amplitude}$ = 0.05 V, $t_{pulse\ width}$ = 0.05 s, $t_{pulse\ period}$ = 0.2 s). (C) Corresponding calibration plots of peak current versus the logarithm of the concentration of TNF-α using (a) ALP-Ab_2-GNPs/PSA and (b) ALP-GNPs-Ab_2 as labels.

gen, more ALP molecules were immobilized at the sensor than that of using ALP-Ab_2-GNPs as the labels, and more electroactive p-NP was obtained by the hydrolysis reaction near the sensor surface which resulted in a stronger signal than that based on the labels of ALP-Ab_2-GNPs.

3.3. Specificity and stability of the immunosensor

Specificity is an important criterion for analytical methods. Some proteins such as carcinoembryonic antigen, bovine serum albumin and human IgG were used to evaluate the specificity. The current values for each interfering substance at a concentration of 30 ng/mL in the presence of 5 ng mL^{-1} of TNF-α were used as an indicator for the selectivity of the sensor in comparison with the TNF-α alone. The ratios of currents with interfering substance to pure TNF-α were 1.027, 0.991 and 1.083, respectively. The results showed that no interference was observed under the experimental conditions. The intra-assay precision was estimated by testing the TNF-α for seven replicate measurements. The inter-assay precision or the fabrication reproducibility was estimated. The relative standard deviations (R.S.D.) of the intra- and inter-assay were 5.7% and 8.1% at the TNF-α concentration of 5 ng/mL, respectively. When the immunosensor was stored in the refrigerator at 4 °C, more than 95% of its initial response was retained for over 1 month. After the biosensor was used for three times, the analytical performances did not show any obvious change. The results showed that the sensor had a good stability, which also suggested that the PANA was an excellent matrix for the immobilization of protein.

3.4. Application of the immunosensor in human serum

The feasibility of applying the sensor in clinical systems was investigated via analyzing real samples, and comparing with the ELISA method. The comparison between the results of the proposed immunosensor and the ELISA method was shown in Table S1 (supporting information). The relative deviations of the two methods were from 7.8% to 9.8%. It obviously suggested that there was no significant difference between the results given by two methods. Therefore, the proposed immunosensor could be reasonably applied in the clinical determination of TNF-α.

4. Conclusion

This paper described the combination of electrochemical immunosensor that used biocompatible PANA film as the platform to immobilizing the immunosensor probe, and the ALP functionalized GNPs/PSA as the novel label for the sensitively detecting of a vital antigen of TNF-α. After the sandwich-type immunoreaction, the conjugated enzyme could hydrolyze the substrate of p-NPP to corresponding electroactive species. And the electrochemical signal had a linear relationship with the concentration of antigen in the range of 0.02–200.00 ng/mL with low detection limit of 0.01 ng/mL. Therefore, a novel immunoassay method was put forward with enhanced performance, and the developed protocol could be easily extended to other protein detection in clinic determination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.03.025.

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