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Ultrasensitive electrochemical immunoassay based on cadmium ion-functionalized PSA@PAA nanospheres

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ABSTRACT

A simple strategy for sensitive detection of human IgG using cadmium ions-functionalized polymer nanospheres as the label is presented. The polymer nanosphere consisted of hard poly-styrene core and biocompatible poly (acrylic acid) (PAA) shell. The carboxyl groups of hydrophilic shell were used to chelated with the cadmium ions, and then conjugate with antibody (Ab_2) to fabricate metal ions marked bioconjugates as the label in immunoassay. For constructing the matrix of the immunosensor, the PAA-functionalized carbon nanotubes were used to modify disposable screen printed electrodes for the immobilization of antibody (Ab_1). After sandwich immunoreaction, differential pulse voltammetry was used to oxidize the conjuncted cadmium for the detection of antigen. The obtained results provided a linear response range from 0.1 to 35.0 pg/mL human IgG with a lower detection limit of 0.06 pg/mL, which is prominently improved in comparison with conventional immunoassay. The usage of the chelation reaction offers a simple and convenient route for the preparation of metallo-immunoassay labels, and also avoids the complicated and time-consuming dissolving of metal component for ultrasensitive determination. This approach is expected to have wide applications in protein diagnostics and bioanalysis in the future.

1. Introduction

Sensitive quantitative detection of protein biomarkers is critical to many areas of biomedical research and diagnostics (Smith et al., 2007), proteomics (Hood, 2003), and systems biology (Kitano, 2002). Particularly, in the systems of various diseases, the development of sensitive, rapid and low cost immunoassay method has become a central task due to the increasing need in true, in-time and expedient diagnoses (Huang et al., 2008). In various detections, electrochemical technique is well recognized because of the high sensitivity, inherent simplicity, reliability, selectivity, and low cost (Hansen et al., 2006a,b; Wang et al., 2006). For obtaining the excellent results, various electroactive labels were found and applied in immunoassay systems, such as metallo-immunoassay and enzyme-link immunoassay. Metallo-immunoassay is the immunoassay using metal-based labels, including quantum dots (QDs) (Hansen et al., 2006a,b; Wang et al., 2003, 2008), metal particles (Rijiravanich et al., 2008; Ambrosi et al., 2007), metal ions (Wang et al., 1998; Hayes et al., 1994), organometallics (Rapicault et al., 1996; Bordes et al., 1997), coordination complexes (Xu, 1997; Blackburn et al., 1991) and so on. Among them, QDs are the most-studied nanomaterials as electroactive labels for the assay of proteins and DNA (Hansen et al., 2006a,b; Wang et al., 2003, 2008; Gill et al., 2008). These quantum dots exhibited sharp and well-resolved stripping voltammetry signals as the metal components has well-defined oxidation potentials. Recently, Liu developed a multi-QDs functionalized silica nanoparticle-based electrochemical amplification platform which dramatically enhanced the intensity of the signal and led to ultrasensitive detection (Chen et al., 2009). Zhu reported a sensitive immunoassay based on QDs nanocrystals as electrochemical and fluorescent tracers (Cui et al., 2007). Although great achievement has been obtained in this field, the finding of more sensitive and convenient assay still attracts increasing interest for improving the behavior of bioassays.

Poly (acrylic acid) (PAA), a water-soluble polymer bearing carboxylic groups along the macromolecular chain, represents the water-soluble multifunctional material with the ability to associate with a variety of multivalent metal ions in solution (Iatridi and Bokias, 2008; Song et al., 2003; Çaykara and İnam, 2003; Yokoi et al., 1986; Kavlak et al., 2003, 2004; Sheeney-Haj-Ichia et al., 2004; Deng and Ting, 2005; Bassaid et al., 2008; Benegasa et al., 1998; Wiedmer et al., 2000), and the sensitivity to external stimuli (Iatridi and Bokias, 2008). Base on the above property, cadmium sulfide (CdS) hollow spherical particles with different

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sizes have been fabricated using core/shell fabrication method with poly-(styrene-acrylic acid) (PSA) latex spheres as guided template (Song et al., 2003). At the same time, PAA has the capability to make function with proteins (Kazakov et al., 2001; Zhou et al., 2007). Mattiasson studied the effect of the conformational state of the polymer coil on the properties of antibody-PAA conjugates (Kazakov et al., 2001). It is also known that surface grafting of PAA is a versatile approach to improve the surface properties of materials. The core-shell microspheres with poly-styrene (PS) core and PAA shell have been synthesized which combined the advantages of the rigidity of the core and the biocompatibility of the soft shell and could be more easily used in aqueous media (Meng et al., 2007). And the functional groups have the capability for metal ion chelation and biomolecular immobilization. Therefore, these functional core-shell spheres are expected to have potential applications in immunoassay.

In this paper, PSA@PAA nanospheres were synthesized, and cadmium ions were combined with PSA@PAA nanospheres by chelation reaction. Furthermore, the hybrid nanospheres were used as a label for the determination of human IgG. After sandwich immunoreaction based on the matrix of PAA functional carbon nanotube modified screen printed electrode, the quantity of immuno-conjuncted antigen could be detected by measuring the voltammetric response of the immobilized Cd^{2+}. To the best of our knowledge, this is the first time that cadmium ions marked PSA@PAA nanospheres were used as a label in immunoassay. Otherwise, compared to conventional QD-based immunoassays, this protocol offers several advantages, especially the elimination of the dissolve process and direct redox in PBS, which greatly reduce time-cost and obtain an ultrasensitive detection. It opens a new door to apply highly sensitive electrochemical immunoassays for potential clinical applications.

2. Experimental section

2.1. Materials and apparatus

Goat anti-human IgG (Ab_1), human IgG (HIgG) (Ag), monoclonal mouse anti-human IgG (Ab_2) and C-reactive protein (CRP) were purchased from Zhengzhou Chuangsheng Biochemical Reagents (Zhengzhou, China). Lyophilized bovine serum albumin (BSA, 99%), Tween-20, Acrylic acid (AA), poly (acrylic acid) (PAA, 35%, w/w in water, MW = 100,000), Poly (diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW = 200,000–350,000), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were from Sigma/Aldrich. Styrene (St) with analytical grade was purchased from Beijing Chemical Reagent Factory (Beijing, China) and was purified before used. Cadmium chloride ($CdCl_2$) and $K_2S_2O_8$ were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Multi-walled carbon nanotubes were purchased from Nanoport. Co. Ltd. (Shenzhen, China), and were treated with sonication in mixed acid $V_{HNO_3} : V_{H_2SO_4} = 1 : 3$ for 6 h. The resulting CNTs were washed repeatedly with distilled water by centrifugation until pH was ~7. Carbon ink (CH-1) and silver conductive ink (BY9700H) were purchased from Shanghai Baoyin Electronic Materials Ltd. (Shanghai, China). 0.1 M PBS with different pH values was prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.1 M NaOH and H_3PO_4. 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 modified screen printed electrodes (SPE) 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 vs. SCE) of the redox couple and perturbation potential of 5 mV. The morphologies of the as-prepared samples were characterized by an S-3000 N (Hitachi, Japan) scanning electron microscope (SEM). The sample for SEM analysis was prepared using the vertical deposition technique to assemble the polymer nanospheres on the ITO coated glass. The ITO was then dried under ambient conditions.

2.2. Fabrication of screen printed electrode

SPEs were prepared using silk printing technique, coated glass strips with silver and carbon inks successively. Firstly, silver ink was printed onto the glass and supported as conductor, and then carbon ink was printed on the silver layer. The film was heated at 60 °C for ca. 5 h to drive off the solvents from the applied paste. The resulted electrodes were designed as 3 mm diameter working area by insulating tape. SPEs were cleaned by washing with PBS and dried at room temperature before use. The used SPE was facile, low cost and disposable, which had potential application in clinic chemistry.

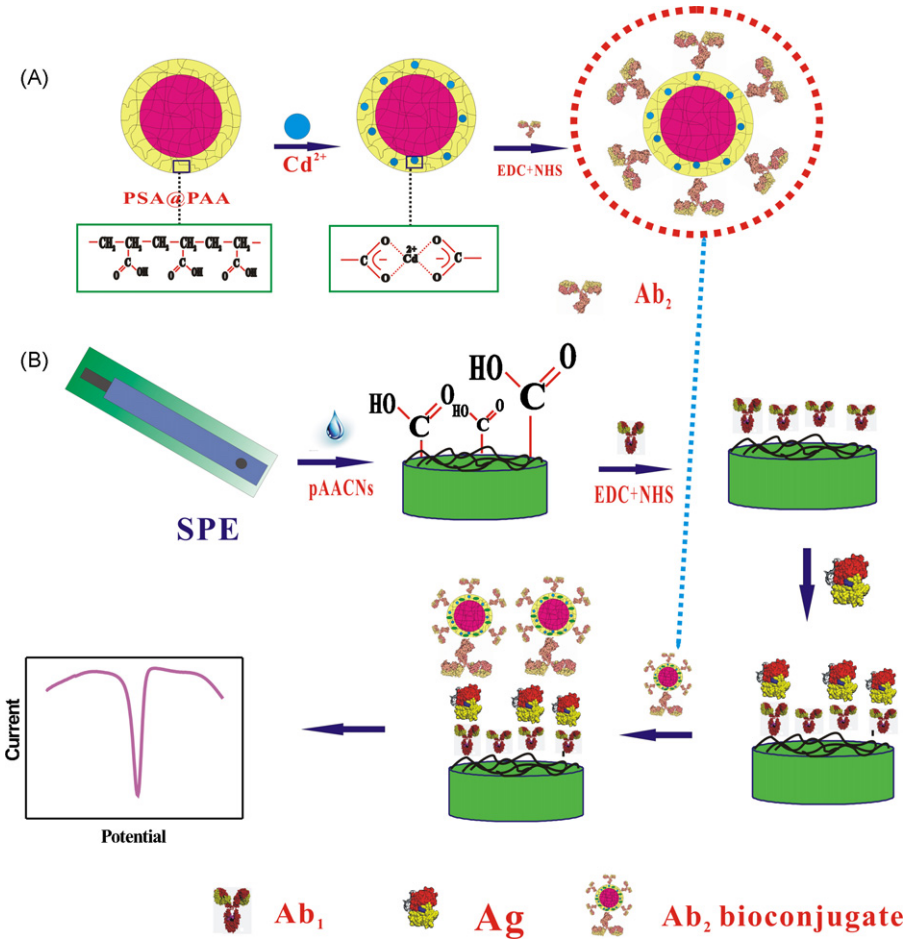
2.3. Preparation of PS and PSA@PAA

The PSA@PAA nanospheres were prepared by an emulsifier-free emulsion polymerization method (Meng et al., 2007; Xiao et al., 2004). A mixture of styrene (4.136 g) and AA (0.900 g) was dissolved in 180 mL of deionized water in a 250-mL three-necked round-bottom flask equipped with a condenser, a nitrogen inlet, and a thermometer. Nitrogen was bubbled into the stirring mixture for 40 min to remove oxygen. Polymerization was initiated by adding $K_2S_2O_8$ (0.200 g, 10 mL) into the mixture at 70 °C. The reaction was proceeding for 8 h at 70 °C under stirring. PAA shell was fabricated on the above-prepared core seeds by a seed polymerization method. After 1.500 g of AA (10 mL) was added, polymerization continued for 15 h under stirring. By this process, the reaction manner was in graft polymerization due to living radicals on the surfaces of polymerizing core spheres. The obtained nanospheres were purified by repetitive centrifugation and redispersed in the same volume aqueous solution. PS nanospheres were prepared via similar process without acrylic acid.

2.4. Fabrication of Ab_2-PSA@PAA/Cd^{2+} Conjugate

The fabrication process is shown in Scheme 1A. 0.5 mL PSA@PAA solution was dispersed in 9.5 mL pure water containing 0.1 mM cadmium chloride under stirring. When PAA is mixed with Cd^{2+} in aqueous solution, the coordination of the carboxyl anions with Cd^{2+} leads to the formation of a PAA-Cd^{2+} complex (Iatridi and Bokias, 2008; Kavlak et al., 2003, 2004; Sheeney-Haj-Ichia et al., 2004; Deng and Ting, 2005; Bassaid et al., 2008). The Cd^{2+} were adsorbed for 5 h under stirring with discontinuous strong ultrasonic to ensure stabilized chelation, and then the PAA-Cd^{2+} complex spheres were separated by centrifugation to remove the unbound and physically adsorbed Cd^{2+}. It was reported that coordination number of similar poly (MA-*alt*-AA)-Cd^{2+} complex appeared as 4, that is to say, one metal ion is coordinated by four chelating groups (Kavlak et al., 2004; Deng and Ting, 2005; Bassaid et al., 2008).

The obtained hybrid was dispersed in 10 mL aqueous solution to obtain a homogeneous dispersion. This dispersion was then mixed



Scheme 1. (A) Assembling procedure of Ab_2-PSA@PAA/Cd^{2+} bionanosphere. (B) The analytical procedure of immunoassay using bioconjugates as label.

with 1 mL aqueous solution containing 40 mM EDC and 40 mM NHS under stirring for 50 min at room temperature. The resulted mixture was centrifuged at 14,000 rpm for 11 min, and the supernatant was discarded. The water washing was repeated to remove excessive EDC and NHS. Secondary antibody of mouse anti-human IgG (0.2 mg/mL, 1 mL) was added to the mixture and stirred overnight at room temperature. The reaction mixture was then centrifuged with 14,000 rpm at 4 °C, and supernatant was removed. After that, 10 mL aqueous solution was added into the obtained solid conjugate and the mixture was centrifuged again with 14,000 rpm at 4 °C and this step was repeated for six times to insure that all free antibody had been removed. The obtained hybrid was dispersed in 10 mL aqueous solution with gently mixing, blocked by 1 mL of 1% BSA solution for 30 min at room temperature, and centrifuged to remove excessive BSA. The collected complex was redispersed into water, and stored at 4 °C.

2.5. Preparation of PAA/PDCNT

The purified CNTs were functionalized with PDDA according to the following procedures: 5 mg CNT was dispersed into 5 mL 0.25% PDDA aqueous solution containing 0.5 M NaCl, and the resulting solution was sonicated for 30 min to obtain a homogeneous black suspension. Residual PDDA polymer was removed by high-speed centrifugation and the product was rinsed with water for at least three times. The collected hybrid was redispersed into water containing 0.25% PAA and 0.5 M NaCl (10 mL), and the resulting dispersion was subsequently sonicated for 20 min and a homogeneous black suspension could be obtained. Residual PAA polymer was removed by high-speed centrifugation and the product was rinsed with water. The obtained solution of PAA/PDCNT was sonicated for 3 min before it was modified on the SPE.

2.6. Detection of HIgG using Ab_2-PSA@PAA/Cd^{2+} labels

The on-chip immunoassay protocol was shown in Scheme 1B. The 5 μL of 5.0 mg/mL PAA/PDCNT solution was dropped onto the SPE and dried for some times. For the attachment of the primary antibody, 30 μL freshly prepared solution of 400 mM EDC and 100 mM NHS were added onto the modified SPE, and washed off after 10 min. Then the modified SPE was immediately incubated in 30 μL 0.2 mg/mL goat anti-human IgG in PBS for 3 h at 35 °C. After that, the electrode was obturated in a chamber with saturated water vapor to avoid sample evaporation. The electrode was then washed with Tween-20 (0.05%) and pH 7.0 PBS. The Ab_1/PAACNT/SPE sensor was incubated in 20 μL 2% BSA and 0.05% Tween-20 for 1 h at 35 °C and washed with 0.05% Tween-20 and PBS buffer. Then the sensors were stored at 4 °C while not in use.

The detection process was depicted as follows. After incubating the immunosensors in solutions containing various concentrations of HIgG for 50 min at 35 °C, followed by washing with pH 7.0 PBS, the resulting sensors were submerged in solution containing Ab_2-PSA@PAA/Cd^{2+} for 50 min at 35 °C. After rinsing thoroughly with pH 6.5 PBS to remove the unbound bionanospheres, the SPE was immersed in an electrochemical cell containing 10 mL of pH 6.5 PBS for DPV scanning from −1.3 to −0.4 V (DPV: $E_{amplitude}$ = 0.05 V, $t_{pulse\ width}$ = 0.05 s, $t_{pulse\ period}$ = 0.2 s).

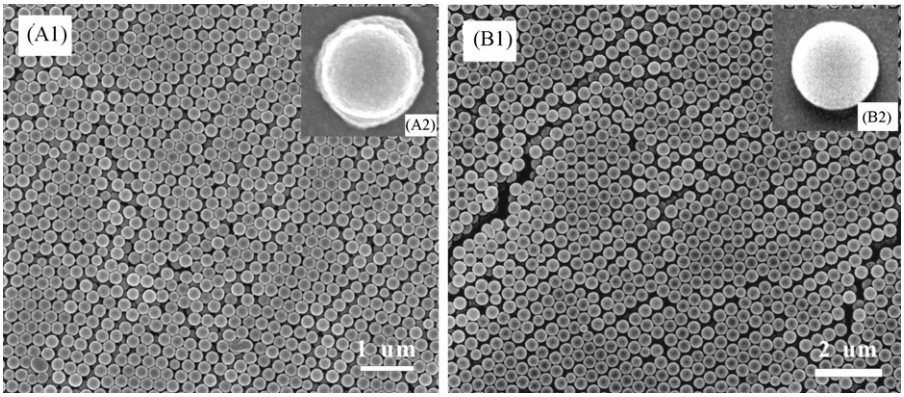


Fig. 1. SEM images of PSA@PAA (A1) and the magnifier (A2); PS (B1) and the magnifier (B2).

3. Results and discussion

3.1. Characterization

The morphology and size of the spheres was characterized by scanning electron microscopy (SEM) technique. The SEM images show that the nanospheres are quite uniform in size (Fig. 1). The prepared PSA@PAA nanosphere has an average diameter of 250 nm and a clearly visible shell structure of about 15 nm (Fig. 1A) indicating the successful surface functionlization of PS sphere which can act with both the metal ions and biomolecules. Compared to PSA@PAA nanosphere, the obtained PS nanosphere shows the diameters of 400 nm and slippery surfaces (Fig. 1B). The results were consistent with the previous reports (Meng et al., 2007; Xiao et al., 2004).

Electron impedance spectroscopy (EIS) was an effective technique to monitor the surface features. The spectra comprised of a semicircle portion and a linear portion, the semicircle portion of higher frequencies corresponds to the electron-transfer limited process, and the linear part of lower frequencies corresponds to the diffusion process. The semicircle diameter is the characterization of the electron-transfer resistance. Fig. 2A shows the Nyquist plots of EIS. At a bare SPE, the redox process showed an electron-transfer resistance of 800 Ω (curve a). The PAA/PDCNT-modified SPE showed a much lower resistance (curve b), implying that PAA/PDCNT was an excellent electric conducting hybrid which could accelerate the electron transfer. After the antibody molecules were combined covalently onto the modified surface, the electron-transfer resistance increased (curve c), suggesting that the immobilized antibody blocked the electron exchange between the redox probe and the electrode. The results also showed that the PAA/PDCNT-modified SPE can effectively immobilize biomolecules with high stability.

3.2. Optimization of assay conditions

Fig. 2B depicted the typical CV response of the immunoreacted SPE in 0.1 M PBS. After Ag-Ab_1/PAACNT/SPE was incubated with polymer nanospheres without Cd^{2+} as the label, no special voltammetric response was observed (curve a). However, after Ag-Ab_1/PAACNT/SPE was incubated with Cd^{2+} labeled nanospheres solution, a well-defined redox peak at −0.9 V appeared, showing the presence of the conjunct cadmium ions. The small voltammetric peak observed during the cathodic sweep at −1.06 V was ascribed to the reduction of Cd(II) to Cd(0), whereas the intense peak observed at −0.77 V during the reversal anodic sweep corresponded to the oxidation of Cd(0) to Cd(II). It was obvious that the anodic peak was sharp and more evident than that of cathodic peak. To achieve high sensitivity, the anodic peak current was used to quantify the concentration of HIgG.

In addition, the reaction conditions including incubation time and temperature (Tang et al., 2004) affected the immunoassay. The effect of incubation time on DPV peak current was shown in Fig. 3A. With the increase in the incubation time, the electrochemical response of the immobilized label increased and then reached a plateau at about 50 min. Therefore, to maximize the signal and minimize the assay time, 50 min was chosen as the optimal incubation time. Fig. 3B showed the effect of incubation temperature on amperometric response. The results suggested that the maximum response occurred at incubation temperature of 35 °C. The lower responses at other temperatures were attributed to the lower reaction rate at lower temperatures and the instability of labeled spheres or reagents at higher temperatures. Therefore, 35 °C was selected as incubation temperature for the immunoassay using the labeled nanosphere solution diluted with the aqueous solution at a volume ratio of 4:1.

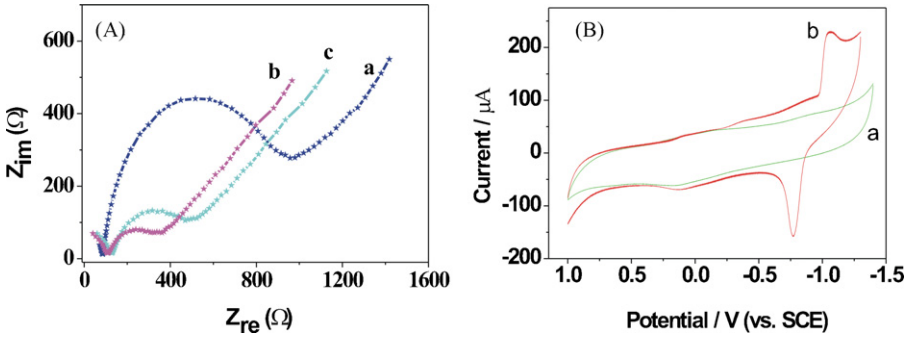


Fig. 2. (A) EIS of (a) SPE, (b) PAACNTs/SPE, (c) Ab_1/PAACNTs/SPE in 2.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.10 M KCl. (B) Typical cyclic voltammograms of immunoreacted biosensor using labels of (a) PSA@PAA-Ab_2, (b) Cd^{2+}/PSA@PAA-Ab_2 in 0.1 M pH 6.5 PBS. Scan rate: 100 mV/s.

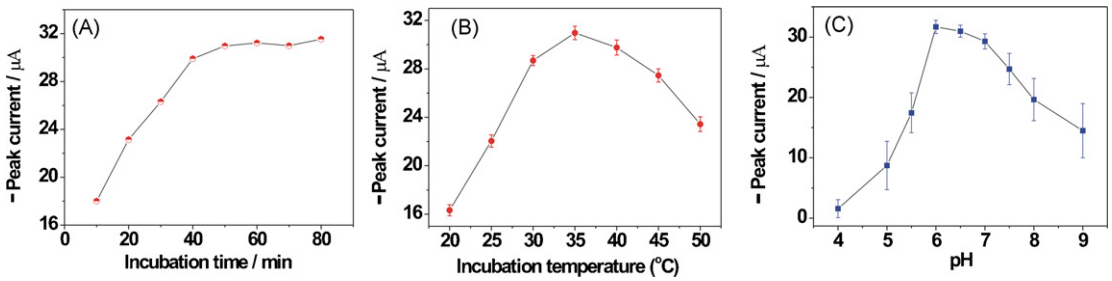


Fig. 3. Effects of (A) incubation time, (B) incubation temperature, and (C) pH of detection solution on peak currents of DPV for 10 pg/mL HIgG.

The acidity of the solution greatly affected the electrochemical behavior of the sensor. Fig. 3C shows the effect of pH of the electrolyte on the current of the immunoassay over a pH range from 4.0 to 9.0 at 5 pg/mL antigen. The results indicated that the excellent pH range was between 6.0 and 7.0. With a lower pH or a higher pH value, the weakened chelate effect will make the signal decrease. So pH 6.5 PBS was selected for immunoassay.

Usually, it is important for the oxidizing signals of the cathodic polarization at a certain potential. However, herein, the response of labeled cadmium ion at a SPE was not affected by electrochemical pretreatment at cathodic polarization with DPV sweep from −1.4 to −0.4 V. Particularly, the HIgG detection was not affected by the oxygen background, which could further simplify the process for the immunoassay.

3.3. Analytical performance

PAA/PDCNT/SPE-based electrochemical immunosensors were used to detect the concentration of HIgG via Cd^{2+} functionalized bioconjugate. Fig. 4A displays typical well-defined voltammetric signals resulting from the oxidation of labeled nanospheres with increasing the concentrations of HIgG. The resulting calibration plot of the peak currents versus C_{HIgG} was linear over the range of 0.1–35.0 pg/mL and was suitable for quantitative application. The detection limit was estimated to be 0.06 pg/mL (0.372 fM) (by the "$S/N = 3$" rule) as shown in Fig. 4B. Compared with other IgG electrochemical sensors reported in the literatures (Ambrosi et al., 2007; Cui et al., 2007, 2008; Dequaire et al., 2000; Selvaraju et al., 2008; Zhang et al., 2008; Zhong et al., 2009; Chu et al., 2005; Das et al., 2006; Wang et al., 2004), this detecting approach exhibited a much lower detection limit.

The feasibility of applying the sensor in clinical systems was investigated via analyzing real samples, and comparing with the ELISA method. A series of HIgG concentration in serum samples were prepared by diluting with PBS. The comparison between the results of the proposed immunosensor and the ELISA method was shown in Table 1. The relative deviations of the two methods were from −6.3 to 10.5%. It obviously suggested that there was no significant difference between the results given by two methods. Therefore, the proposed biosensor could be reasonably applied in the clinical determination of HIgG.

Table 1
The comparison of HIgG levels in serum given by electrochemical assay and ELISA method.

Serum samples	1	2	3	4	5
Immunosensor (pg/mL)[a]	0.221	0.840	2.45	8.90	25.2
ELISA[a]	0.200	0.900	2.70	9.50	23.5
Relative deviation (%)	10.5	−6.7	−9.2	−6.3	7.2

[a] The average value of three successive determinations.

3.4. Specificity, precision and stability of the immunosensor

It is necessary to check the specificity, a significant character, for the fabricated immunoassay method. Here, the selectivity was examined by comparing the electrochemical responses of 10 pg/mL pure HIgG and a same solution containing additional interferential substance of 40 pg/mL, such as C-reactive protein (CRP), goat IgG or bovine serum albumin (BSA). Ratios of currents for a mixture to pure HIgG were 0.988, 1.034 and 1.091, respectively. In real sample analysis, the specificity was also tested in the systems containing known interfering substances. The interfering substances-containing mixtures were obtained by adding 40 pg/mL of other proteins such as CRP, goat IgG or BSA into the real samples with HIgG concentration of 9.5 pg/mL, respectively. Ratios of signals for the mixture to the real sample were 0.973, 1.057, and 1.108, respectively, which were satisfactory in the determination of real samples. This means no interferential phenomena in the presence of above interfering substance. So the prepared sensor possesses acceptable selectivity. The results of six repeated detection of the

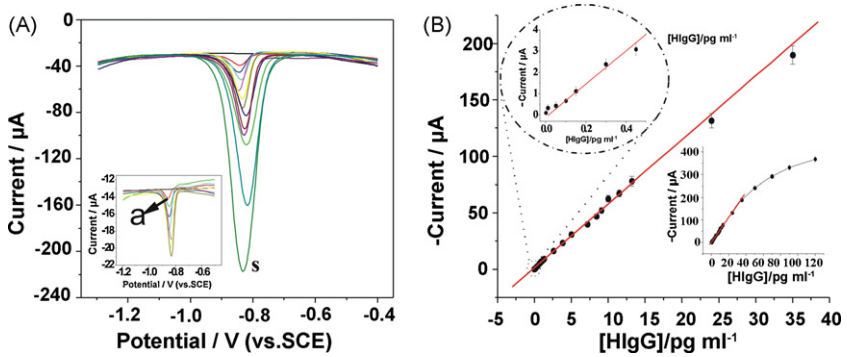


Fig. 4. (A) Typical electrochemical DPV curves corresponding to Cd^{2+} oxidizing of immunoconjugate with increasing concentration of the HIgG (from a to s, 0.1–35.0 pg/mL, respectively). The inset signals were corresponding to the concentration of HIgG from 0.1 to 0.85 pg/mL. Experimental conditions: in pH 6.5 PBS and scan from −1.3 to −0.4 V (DPV: $E_{amplitude} = 0.05$ V, $t_{pulse\ width} = 0.05$ s, $t_{pulse\ period} = 0.2$ s). (B) Calibration curves of the immunosensor for HIgG determination in pH 6.5 PBS. Inset: amplification of the calibration curve in low (top left) and high (right) concentration of HIgG.

HIgG were used to character the intra-assay precision. And the relative standard deviation was 6.2% corresponding to 10 pg/mL HIgG. Thirty cycle scans were carried out in the potential range from −1.4 to −0.4 V in pH 6.5 PBS, a 5.8% decrease in the initial response was observed, which indicated that the sensor had good stability. When the sensor was stored at 4 °C under dried circumstance, the 87.7% signal of its initial response was retained after twenty days. The stablility of the prepared sensor may be attributed to the stable chelation between cadmium ions and PAA. And these results further indicated that PAA/PDCNT was suitable for the immobilization of biomolecules and could retain the bioactivity due to the strong interaction with biomolecules and the better biocompatibility. Thus, the developed immunosensor has potential application for the determination of HIgG in clinical systems.

4. Conclusion

An ultrasensitive and simple electrochemical immunosensor was proposed and applied in typical HIgG detection, in which Cd^{2+}/PSA@PAA chelating hybrid was used as labels to obtain the signal by oxidizing the metal on disposable SPE. After the sandwich immunoreaction, the signal of introduced metal ion labels provided high sensitivity and precision with a linear response range of 0.1–35.0 pg/mL and a lower detection limit of 0.06 pg/mL. The sensitivity of the immunoassay system is superior to those of previously reported electrochemical methods. And the sensor exhibited satisfactory response for the analysis of human serum samples. The simple, sensitive and disposable SPE-based electrochemical immunosensor has potential application in detecting HIgG for clinical systems. The labels of chelate compound could be also extended to other metal ions for multi-protein detection.

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