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Gold nanoparticles and polyethylene glycols functionalized conducting polyaniline nanowires for ultrasensitive and low fouling immunosensing of alpha-fetoprotein

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ABSTRACT

An ultrasensitive biosensor for alpha-fetoprotein was developed based on electrochemically synthesized polyaniline (PANI) nanowires, which were functionalized with gold nanoparticles (AuNPs) and polyethylene glycols (PEG). The prepared PEG/AuNPs/PANI composite, combining the electrical conductivity of the AuNPs/PANI with the robust antifouling ability of PEG, offered an ideal substrate for the development of low fouling electrochemical biosensors. Alpha-fetoprotein (AFP), a well-known hepatocellular carcinoma biomarker, was used as a model analyte, and its antibody was immobilized on the PEG/AuNPs/PANI for the construction of the AFP immunosensor. Using the redox current of PANI as the sensing signal, in addition to the good biocompatibility of PEG/AuNPs and the anti-biofouling property of PEG, the developed immunosensor showed improved biosensing performances, such as wide linear range and ultralow detection limit (0.007 pg mL^{-1}). More importantly, it is label-free, reagentless and low fouling, making it capable of assaying AFP in real serum samples without suffering from significant interference or biofouling.

1. Introduction

Hepatocellular carcinoma (HCC) has been the third cause of cancer death and the leading cause of mortality among cirrhotic patients (Forner et al., 2012). Over one million patients have been diagnosed with HCC worldwide per year (Kew, 2010; Yang and Roberts 2010). Alpha-fetoprotein (AFP), a 70-kDa glycoprotein, is a biomarker whose serum concentration increases in most HCC patients (Suriapranata et al., 2010), therefore, it has been used for the surveillance and diagnosis of HCC in patients with chronic liver disease (Bertino et al., 2012). Thus, detecting AFP in human serum is of great significance for the diagnosis and management of HCC (Wang et al., 2014a). Various methods have been developed for the detection of AFP, such as chemiluminescence (Fu et al., 2006), enzyme-linked immunosorbent assay (Bi and Liu, 2014), surface plasmon resonance (Chang et al., 2009) and radioimmunoassay (Shafik et al., 2014). Besides, electrochemical immunosensors, possessing high sensitivity, rapid detection, low cost and ease miniaturization, have aroused great interests and have been applied in the detection of tumor markers (Gao et al., 2013; Wang et al., 2014a).

In order to improve the sensitivity of electrochemical immunoassays, a variety of nanomaterials have been utilized for signal amplification in the fabrication of the immunosensors, such as metal nanoparticles (Li et al., 2015a), carbon-based nanostructures (Malhotra et al., 2010), graphene sheets (Lin et al., 2012) and conducting polymers (Bangar et al., 2009). Particularly worth mentioning are the conducting polymers, which possess high electrochemical performance, including the controllable conductivity to increase overall response and good biocompatibility for biomolecules immobilization. As an important member of conducting polymers, polyaniline (PANI) has been widely investigated because of its fast electron transfer dynamics and excellent environmental stability (Zhang and Rutledge, 2012). Moreover, PANI can be employed as a direct electron mediator to further amplify the electrochemical signal. However, PANI performs conductive well just in acidic solution, making it a shortcoming in its biosensing application. To solve this problem, PANI has been integrated with gold nanoparticles (AuNPs), which could shift PANI redox to neutral pH environment (Granot et al., 2005). Additionally, hybrid nanocomposites of PANI with AuNPs have shown improvement in the charge transfer, functionality and stability of PANI-based matrix (Arya et al., 2011; Dey et al., 2012; Lai et al., 2014; Ozdemir et al., 2010). An electrochemical DNA biosensor based on PANI nanofibers modified with AuNPs has

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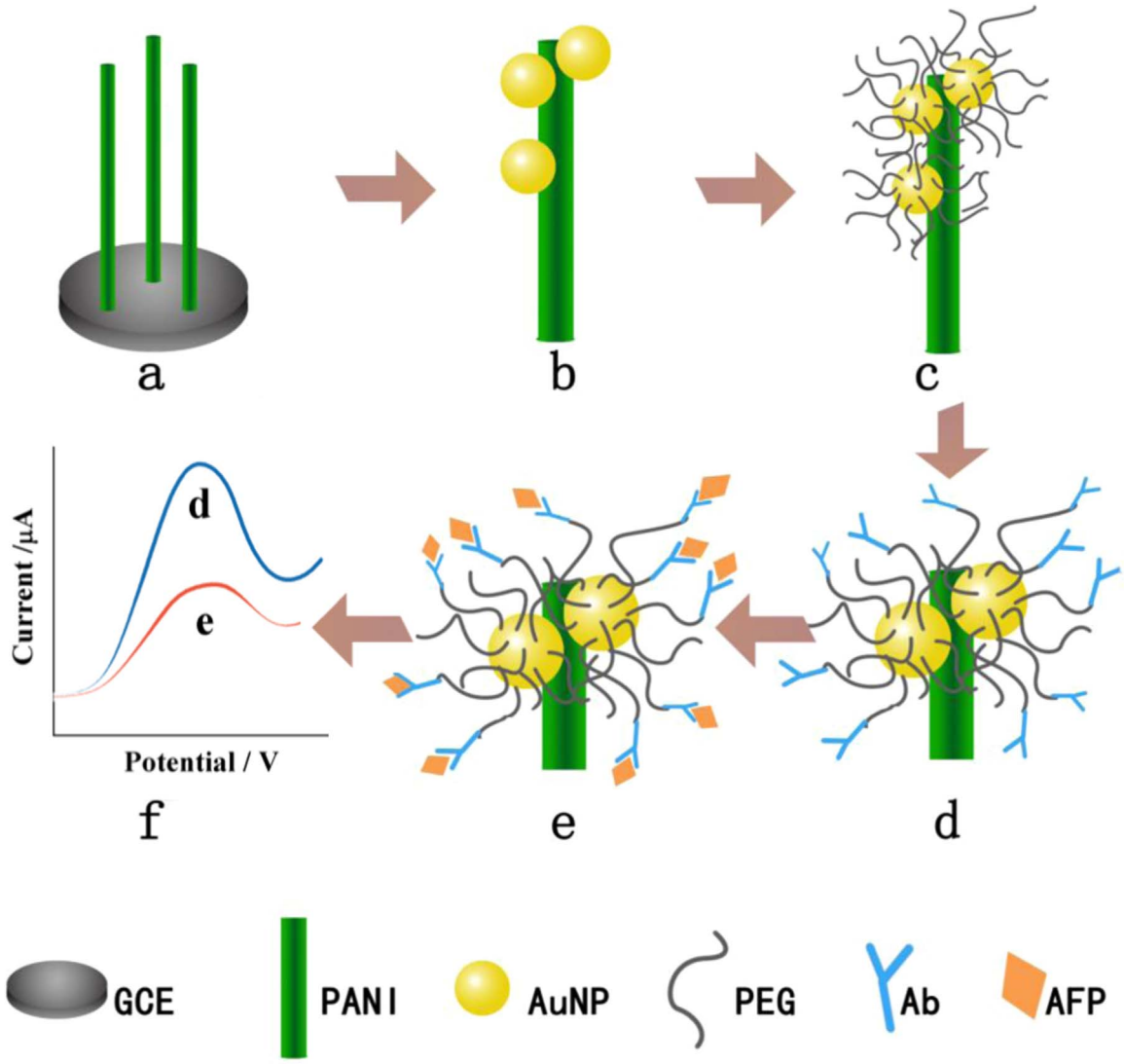

Scheme 1. The fabrication process of the AFP immunosensor. (a) PANI nanowires deposited on the GCE, (b) AuNPs electrodeposition, (c) PEG modification, (d) AFP antibody immobilization, (e) AFP target capturing and (f) DPV current signal recording.

revealed enhanced loading of the DNA probe and wider dynamic range of the DNA sensor (Wang et al., 2014b). An ultrasensitive immunmoassay method has also been developed based on the electrochemical measurement of polyaniline produced by horseradish peroxidase-functionalized gold nanoparticle (HRP-AuNP). Both the signal amplification of the PANI and the electron transfer acceleration of AuNPs on the immunosensor surface greatly improved the sensitivity of the cortisol immunoassay method (Arya et al., 2011). The above reports demonstrated that these nanocomposites possess high conductivity and excellent electroactivity, which have been used as a promising matrix for the immobilization of biomolecules.

Nanostructured PANI, including nanowires, nanorods, nanospheres, and nanotubes, have offered a new foundation to improve their characteristics as they possess very high surface area (Forzani et al., 2004). For instance, owing to higher effective surface area and shorter penetration depth for target molecules, nanostructured PANI has exhibited faster response time and greater sensitivity compared with its bulk counterpart (Zhao et al., 2009). Recently, Sreedhar et al. (2009) have investigated the activity profile of the oxidation of 4-bromothioanisole using PANI nanospheres, nanorods, and nanotubes. The experimental results revealed that nanotubes have the largest activity, which was attributed to the enhanced surface area of PANI nanotubes. However, few previous reports have investigated the application of PANI nanomaterials modified sensor in complex media (human serum), as nanomaterials with a large surface area may cause severe nonspecific protein adsorption or biofouling.

Surface fouling of immunosensors is a ubiquitous and problematic phenomenon, which can significantly reduce the sensitivity and accuracy of quantitative results. Protein fouling, referred to as nonspecific adsorption from natural samples such as serum, is a crucial issue for immunosensing, seriously confining the accurate target detection only in the presence of a protein-resistant background (Pop-Georgievski et al., 2012; Vaisocherová et al., 2008). PEG-based polymers are recognized as one type of the most powerful fouling-resistant materials, which have been applied in a range of biosensors (Eck et al., 2008; Matsuya et al., 2003; Simpson et al., 2011). When PEG was integrated with solid surface, their interactions with cells and proteins could be effectively reduced (Christophis, 2010; Xu et al., 2013) via “steric repulsion” (Meyerbroker et al., 2013), which was an entropic effect caused by the dehydration of flexible polymer chains and the unfavorable change in free energy associated with confinement (Akkahat et al., 2012). Therefore, fabricating a protein-resistant surface, capable of reducing or eliminating nonspecific

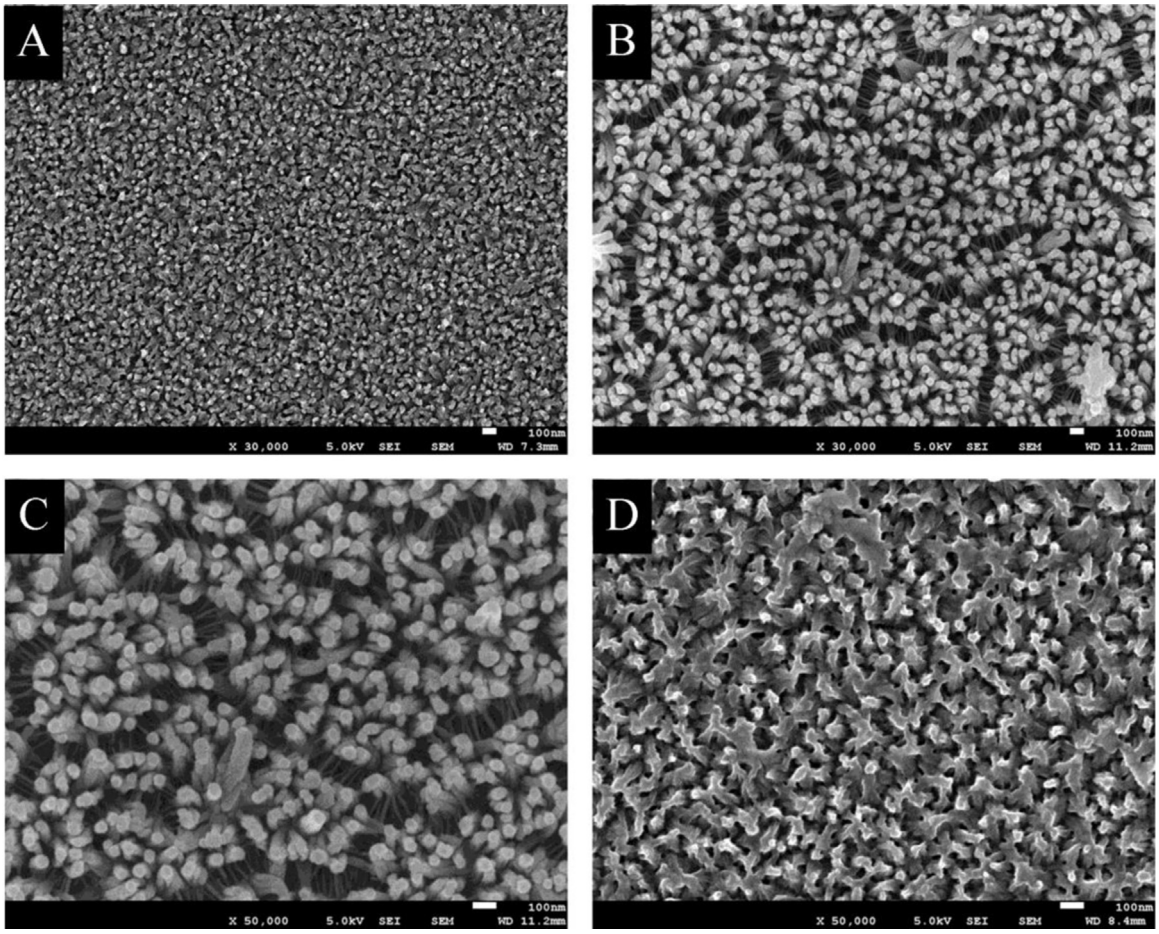

Fig. 1. SEM images of electrodes modified with (A) PANI nanowire arrays, (B, C) AuNPs/PANI and (D) PEG/AuNPs/PANI nanocomposites.

adsorption of proteins, can improve the selectivity and accuracy of the immunosensors.

Herein, an electrochemical immunosensor capable of biosensing in complex samples was designed for the quantitative detection of AFP. The synergetic effect of AuNPs functionalized PANI nanowires was employed to increase the sensitivity of the immunosensor. Electrodeposited AuNPs was integrated with PEG-NH_2, which could be effectively conjugated onto the surface of AuNPs by the interaction between AuNPs and –NH_2 on PEG (Wang et al., 2014c). In this work, PEG-NH_2 as the antifouling material was used both to improve the selectivity of the immunosensor and to immobilize AFP antibodies (Ab). The prepared immunosensor was proved to be a useful tool for the quantitative detection of AFP in human serum with a wide linear range, ultrahigh sensitivity, low detection limit and antifouling ability.

2. Materials and methods

2.1. Regents

4-arm PEG terminated with amino groups (2000 Da) was purchased from Suzhou Nord Derivatives Pharm-Tech co., Ltd. Human serum albumin (HSA), pepsinogen (PG), ɤ-globulin from bovine blood (GLO), trypsinogen (Try), Alpha fetoprotein (AFP) antibody and antigen were obtained from Beijing Xinkezhongjing Biological Technology Co. Ltd (Beijing, China). DNA sequence (5′ GAA CAA AAG GAA GAA AAT C 3′) were supplied by Sangon Biotech (Shanghai, China) Co., Human serum samples were provided by the Eighth People's Hospital (Qingdao, China). Other reagents were of analytical grade. All solutions were prepared with millipore water obtained from a Milli-Q water purifying system.

2.2. Apparatus

Scanning electron microscope (SEM) was used to characterize morphologies and nanostructures of PANI, AuNPs/PANI and PEG/AuNPs/PANI nanocomposites (JEOL JSM-7500F SEM instrument Hitachi High-Technology Co., Ltd., Japan). X-Ray photoelectron spectroscopy (XPS) analysis was measured using a Thermo-VG Scientific ESCALAB 250 spectrometer with a high-performance Al monochromatic source operated at 15 kV. Wetting behaviors of the materials were measured by a contact angle (CA) measuring instrument (JC2000 Shanghai Zhongcheng instrument Co., China). Electrochemical measurements were performed using a CHI660E Electrochemical Analyzer (Shanghai CH Instrument Co., China), with the modified glassy carbon electrode (GCE, diameter 3.0 mm) as the working electrode, a Ag/AgCl electrode and a platinum wire as the reference electrode and the auxiliary electrode, respectively. Samples were prepared on the indium tin oxide (ITO) coated glass for XPS and CA measurements.

2.3. Fabrication of the AFP immunosensor

GCE was polished, washed and electrochemically pretreated in phosphate buffered saline (PBS) (Luo et al., 2007). The AFP immunosensor was prepared step-wise, as shown in Scheme 1. Firstly, PANI nanowire arrays were deposited onto the pretreated electrode by galvanostatic technique in 1 M $HClO_4$ solution containing 0.5 M aniline (Controlling the current density at 0.6, 0.3,

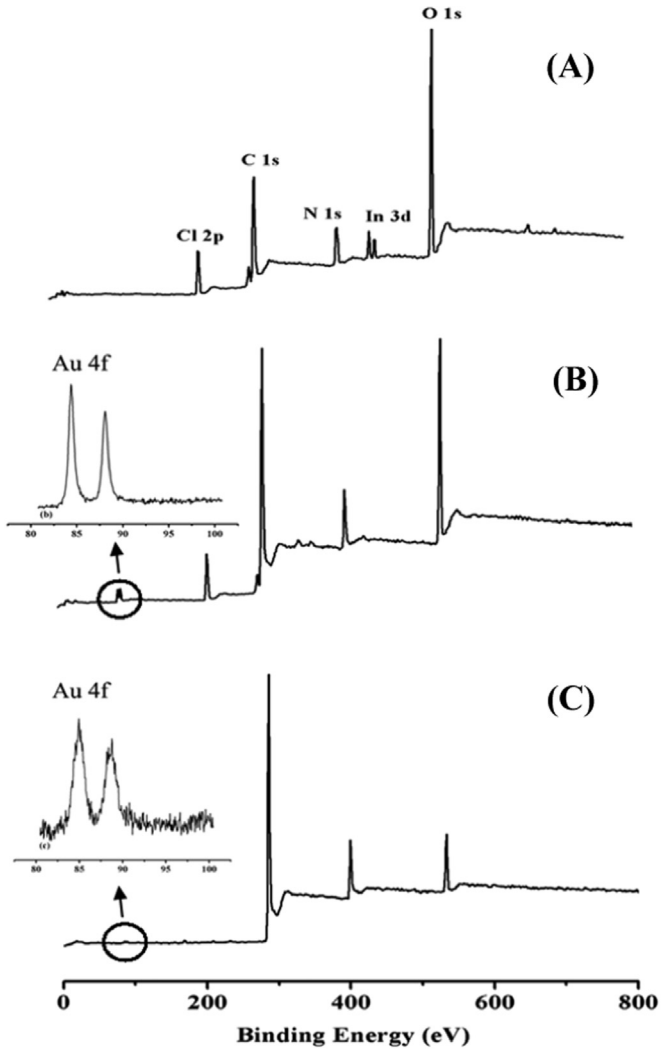

Fig. 2. X-ray photoelectron spectra of (A) PANI, (B) AuNPs/PANI, and (C) PEG/AuNPs/PANI coated Indium tin oxide (ITO) surfaces.

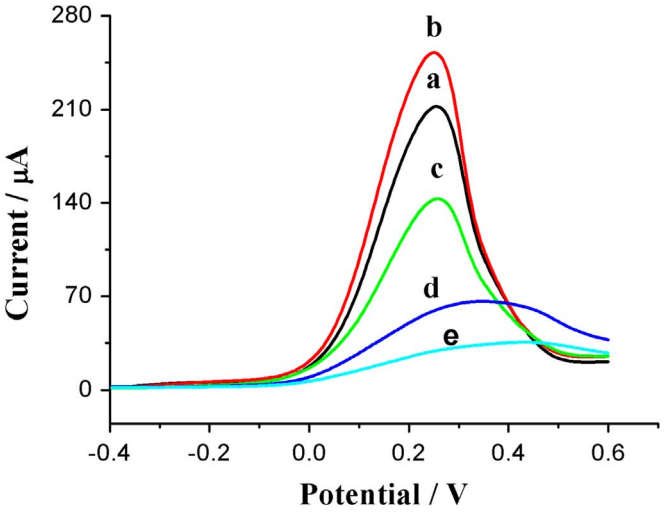

Fig. 3. DPVs measured in PBS (0.2 M, pH 5.7) for (a) PANI, (b) AuNPs/PANI, (c) PEG/AuNPs/PANI, (d) Ab/PEG/AuNPs/PANI, and (e) AFP/Ab/PEG/AuNPs/PANI.

0.15 mA cm^{-2} for 0.5 h, 2 h, 2 h, respectively). Secondly, for the further deposition of AuNPs, the PANI nanowire arrays modified electrode was immersed in a solution containing 50 μM $HAuCl_4$ and carried out electrodeposition at a potential of −0.1 V (vs. Ag/AgCl) for 50 s. Thirdly, the formed AuNPs/PANI electrodes were immersed in 2.0 mL PBS solution (0.2 M, pH 7.4) containing 2.0 mg mL^{-1} 4-armed PEG-amine. A highly nonfouling cross-linked hydrated polymeric network is formed onto the surface of AuNPs/PANI through the interaction between AuNPs and -NH_2 groups of the PEG. Lastly, The PEG/AuNPs/PANI modified electrodes were incubated in PBS solution (10 mM, pH 7.4) containing 0.4 M EDC, 0.1 M NHS, and 5.0 μg mL^{-1} AFP antibody for 3 h. The fabricated AFP biosensor (Ab/PEG/AuNPs/PANI/GCE) was adequately washed with water and stored in PBS at room temperature before use.

2.4. Electrochemical measurements

Electrochemical impedance spectroscopy (EIS) measurements were recorded in a solution containing 0.1 M KCl and equimolar 5 mM [$Fe(CN)_6^{4-/3-}$] at the frequency range from 1 to 100,000 Hz. The amplitude of the applied sine was 5 mV with the current potential set as 0.20 V. To carry out the immunoreaction and electrochemical measurement, the immunosensor was first incubated with AFP standard solutions or serum samples for 60 min at room temperature, followed by washing with PBS and water, and differential pulse voltammetry (DPV) was performed in PBS solution (0.2 M, pH 5.7) to record the peak currents of PANI deposited at the immunosensor for quantitative analysis.

3. Results and discussions

3.1. Characteristics of the modified electrodes

The surface morphologies of electrodes modified with PANI, AuNPs/PANI and PEG/AuNPs/PANI were characterized by SEM. As shown in Fig. 1A, the prepared PANI formed a uniform film on the electrode surface. And the aligned PANI nanowires were all oriented perpendicular to the substrate, with the diameter of 21.62 ± 2.26 nm and the length of 206.1 ± 3.68 nm. As PANI nanowires were uniformly distributed on the whole electrode, they can act as suitable substrate for the loading of other nanomaterials. Fig. 1B and C shows that AuNPs were homogeneously distributed on the PANI nanowires, indicating successful attachment of AuNPs onto the PANI nanowires, especially the nanowire tips. As PANI nanowires contain many terminal amine groups, AuNPs can easily attach to the PANI nanowires through their interaction with the amine groups. More interestingly, PEG containing amine groups can be further attached to the AuNPs/PANI modified surface through their interaction with AuNPs, and the resulted electrode surface shows a cross-linked and porous microstructure, with the diameter of the nanowires increased to 53.94 ± 3.28 nm due to the modification of PEG (Fig. 1D).

In the XPS analysis, the PANI-coated indium tin oxide surfaces showed C 1s, N 1s, O 1s, and In 3d peaks, where the C 1s and N 1s peaks corresponded to PANI (Fig. 2A). After electrochemical deposition of AuNPs, new Au 4f peaks (83.8 eV and 87.7 eV) corresponding to metallic Au° were observed (Fig. 2B), which further confirmed the formation of AuNPs on PANI nanowires (Liu et al., 2014). However, the decrease of Au 4f peaks accompanied with the concurrent increase in the peak intensities of C 1s appeared after PEG modification, demonstrating that PEG has been successfully attached to the surface of AuNPs/PANI.

To characterize the hydrophilicity of different materials, the static water contact angles of PANI, AuNPs/PANI and PEG/AuNPs/PANI modified surfaces were measured. As shown in Fig. S1 and Table S1 (Supporting information), bare ITO without any pretreatment exhibits a moderate level of hydrophilicity with a

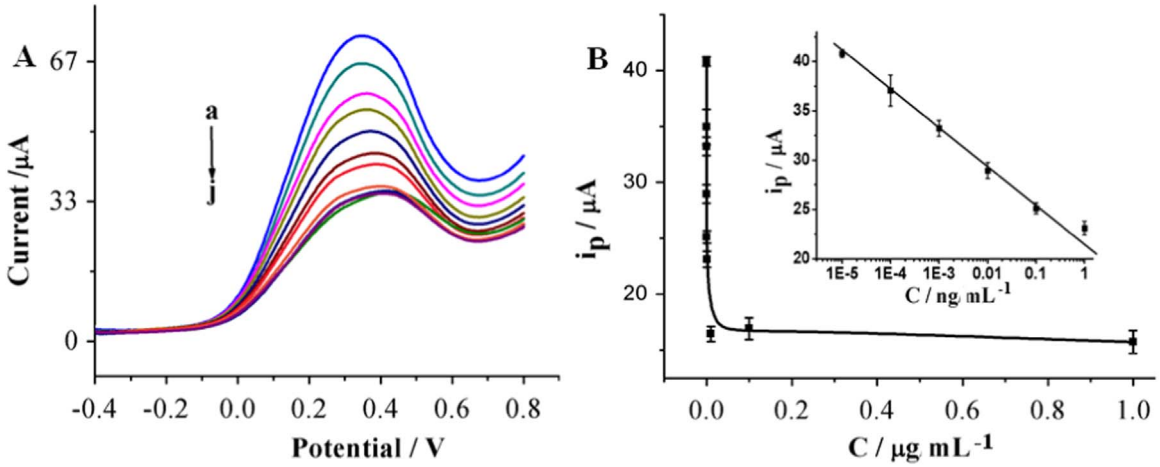

Fig. 4. (A) DPV responses of the immunosensor toward different concentrations of AFP and (B) the dynamic response range of the immunosensor. Fig. A, curve a refers to the blank solution and curves b−j correspond to AFP at the concentration from 10^{-14} to 10^{-6} mg mL^{-1}; Fig. B, the inset is the calibration curve of the immunosensor. Error bars represent the standard deviations of three repeated determinations (n=3).

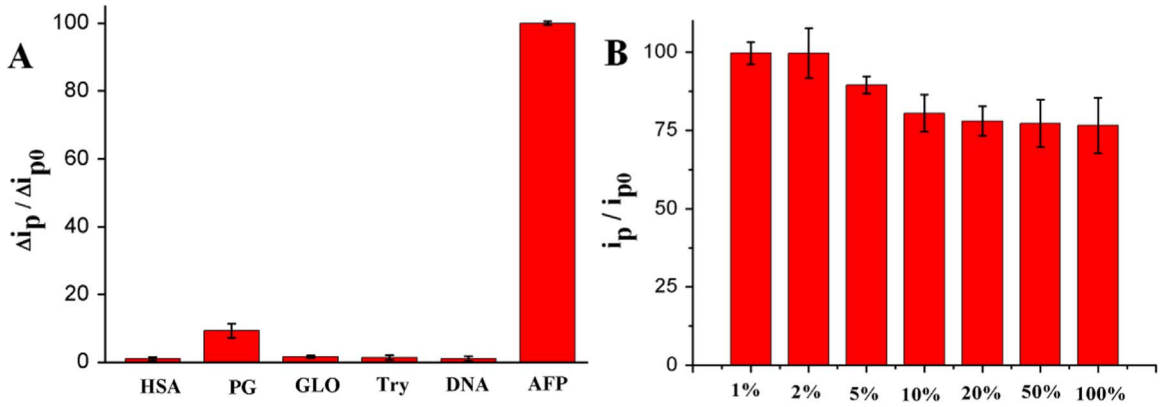

Fig. 5. (A) Responses of the AFP immunosensor to 1.0 μg mL^{-1} HSA, PG, GLO, Try, DNA sequence and 100 pg mL^{-1} AFP antigen, respectively. (B) The antifouling property of the AFP immunosensor in human serum of different concentration (V/V). Error bars represent the standard deviations of seven repeated determinations (n=7).

contact angle of 75.5°, close to the previously reported value of 70° (Abdelsalam et al., 2005). PANI coated ITO surfaces show slightly better hydrophilicity with a contact angle of 55.1°, presumably due to the influence of the amino groups of PANI. As expected, the AuNPs and PEG-grafted ITO substrates show much lower contact angles (25° and 18°, respectively), indicating excellent hydrophilicity. The contact angle of PEG/AuNPs/PANI surface is near the reported values of other antifouling materials in the literature, such as zwitterionic polymer (e.g., poly-MPDSAH, 17°) (Zhao et al., 2011), and poly(β-peptoid)s (17°) (Lin et al., 2011). Thus, the PEG/AuNPs/PANI with super-hydrophilicity may effectively prevent nonspecific adsorption of proteins onto its surface through the hydrophobic interaction, and therefore becoming an ideal substrate for the construction of antifouling biosensors.

3.2. Electrochemical characterization of the immunosensor

In order to characterize the fabrication process of the immunosensor, DPV of different modified electrodes were recorded and shown in Fig. 3. An obvious cathodic peak at 0.26 V appears for the PANI modified electrode (Fig. 3 curve a), corresponding to the transition of leucoemeraldine/emeraldine. As shown in Fig. 3 curve b, the peak current increased after the electrodeposition of AuNPs, as AuNPs are highly conductive and they can act as electron shuttles (He et al., 2015). In contrast, the attachment of PEG led to an obvious decrease of the current response owing to the formation of a less conducting layer. Subsequently, the peak current response further decreased after incubation in AFP antibody and AFP solution, respectively. This is because the immobilization of AFP antibodies and the further binding of AFP targets (formation of antigen-antibody immunocomplexes) will retard the transfer of electrons toward the electrode surface (Liang et al., 2012). The strong antibody-antigen binding, taken place in the electrode surface, will make the electrode surface covered by the insulating biomolecules, which can block the electron transfer and result in peak current decrease and peak potential shifting. Clearly, based on the DPV peak current decrease along with the binding of AFP targets, an immunosensor for the quantitative analysis of AFP can be developed.

3.3. Optimization of experimental conditions

The pH value and incubation time are two important parameters in the immunoreaction. In order to optimize the pH, the immunosensor was tested in a series of PBS with the pH values varying from 5.0 to 6.5. As shown in Fig. S2, the current responses increase with increasing pH value from 5.0 to 5.7 and then decrease rapidly at the higher pH values. As a consequence, PBS of pH 5.7 was chosen as the detection solution for further experiments in this study. This pH value is a compromise condition for the immunoreaction to take place and at the same time keep the PANI considerably electroactive. As for the incubation time, it can be clearly observed that the current responses increased with increasing incubation time and reached a plateau after 60 min,

indicating that the equilibrium of the immunoreaction was established. Therefore, 60 min was selected as the optimum time for all the following incubation steps of the assay.

3.4. Electrochemical response studies

The electrochemical response of the Ab/PEG/AuNPs/PANI modified electrode (immunosensor) has been studied as a function of AFP concentration. The current response of the immunosensor decreased along with the increasing of the AFP concentration (Fig. 4A), which confirmed the successful formation of immunocomplexes between the AFP antigen and antibody. The response range of the sensor is 0.01 pg mL^{-1}–1.0 ng mL^{-1} (Fig. 4B) with a limit of detection (LOD) of 0.007 pg mL^{-1} (3σ, n=10). The LOD is much lower than that achieved by previously reported AFP immunosensors, such as those using localized surface plasmon resonance (LSPR) based on gold nano-mushroom (Li et al., 2015b), surface-enhanced Raman scattering (SERS) based on gold nanoparticle-embedded metal-organic framework (Hu et al., 2014), and enzyme-linked immunosorbent assay (ELISA) based on molecularly imprinted polymers (MIPs) (Bi and Liu, 2014). This low LOD was mainly attributed to the good electron transfer capability of AuNPs/PANI and the excellent biocompatibility of PEG, which can provide a suitable micro-environment for the immobilized AFP antibody to exhibit better binding affinity.

3.5. Specificity and antifouling property of the immunosensor

The selectivity and reproducibility are important parameters of a biosensor for practical applications. To evaluate the reproducibility of the immunosensor, five different electrodes prepared independently were employed for the detection of AFP (100 pg mL^{-1}), and the relative standard deviation (RSD) of the measurements was 6.8%. To evaluate the selectivity of the electrochemical immunosensor toward its target AFP, the signal response of the immunosensor was assessed in various solutions containing HSA, PG, Try, GLO and single-strand DNA sequence, or target antigen (AFP). As shown in Fig. 5A, very small and negligible signal responses to HSA, Try, GLO and DNA were observed, and only PG generated some influence. However, considering the fact that the tested concentration of PG is 10,000 times higher than that of the target AFP, the effect of PG on the assay of AFP in real systems will be very limited. Therefore, the biosensor demonstrated excellent selectivity to its target AFP antigen.

For a biosensor to be practically used for real samples, it is necessary to evaluate its antifouling property in complex biological media, such as human serum. AFP immunosensors were firstly saturated in AFP solution to exclude possible interference from AFP in human serum, and then the immunosensors were incubated in different serum samples to test their anti-biofouling ability. As shown in Fig. 5B, after incubation in 1% and 2% human serum, the current response of the immunosensor only had a minor change of less than 0.3%. After incubation in 5% human serum, the immunosensors retained 89.5% of their initial current responses. More interestingly, even after incubation in the pure serum, the immunosensors still retained about 77.1% of their initial responses, indicating excellent antifouling ability of the AFP immunosensor in complex human serum samples.

3.6. Real sample analysis

In order to evaluate accuracy and feasibility of the developed immunoassay for real sample analysis, the concentrations of AFP in different serum samples were detected using the standard addition method. The results are summarized in Table S2 (before detection, the serum sample 3 was diluted 10 times by 0.01 M pH 7.4 PBS). It is clear that the recovery rate was varied from 102.3% to 104.7%, and the RSD was ranged from 4.78% to 5.04%, which are acceptable for clinical applications in the determination of the disease biomarker levels in serum samples.

4. Conclusion

In summary, an electrochemical immunosensor based on the hybrid composite of PEG/AuNPs/PANI was successfully developed. Combining unique properties of PANI nanowires (inherent electroactivity and good conductivity), AuNPs (high conductivity and biocompatibility) and PEG polymer (excellent biocompatibility and fouling-resistant property), the prepared AFP immunosensor was found to be reagentless (using PANI redox signal, without additional signal reagents), highly sensitive and selective, and most importantly, low fouling in complex serum samples. When functionalized with other biological probes instead of AFP antibody, the biosensing system is expected to be capable of measuring other targets in complex biological samples without suffering from biofouling (nonspecific adsorption).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.06.028.

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