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# One-step synthesis of redox-active polymer/AU nanocomposites for electrochemical immunoassay of multiplexed tumor markers

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ABSTRACT

In this work, a simple and sensitive multiplexed immunoassay protocol for simultaneous electrochemical determination of alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) was designed using redox-active nanocomposites. As the redox-active species, the poly(o-phenylenediamine) (POPD)/Au nanocomposite and poly(vinyl ferrocene-2-aminothiophenol) (poly(VFc-ATP))/Au nanocomposite were obtained by one-step method which $HAuCl_4$ was used as the oxidant. With Au nanoparticles (AuNPs), the nanocomposites were successful to immobilize labeled anti-CEA and anti-AFP as the immunosensing probes. The proposed electrochemical immunoassay enabled the simultaneous monitoring of AFP and CEA in a wide range of 0.01–100 ng $mL^{-1}$. The detection limits was 0.006 ng $mL^{-1}$ for CEA and 0.003 ng $mL^{-1}$ for AFP ($S/N=3$). The assay results of serum samples with the proposed method were well consistent with the reference values from standard ELISA method. And the negligible cross-reactivity between the two analytes makes it possesses potential promise in clinical diagnosis.



## 1. Introduction

The development of materials science has brought significant progress for simultaneous electrochemical determination of multiplex biomarkers (Wilson, 2005; Liu et al., 2014; Harper et al., 2007; Jia et al., 2014; Karimi-Maleh et al., 2013; Tavana et al., 2012; Ensafi et al., 2011). Compared with the traditional materials, nanomaterials have stimulated intense research over past decades due to their potential advantages for electrochemical bioassay, including the excellent biocompatibility, facile synthesis, flexible control over the size, high surface area, good conductivity, and easy modification for biomolecule (Rosi and Mirkin, 2005; Hao et al., 2010; Cosnier et al., 2008; Wang et al., 2008; Jeong et al., 2013; Li et al., 2011; Elyasi et al., 2013; Moradi et al., 2013). Various nanomaterials such as metal nanoparticles, quantum dots, graphene oxide, and magnetic nanoparticles, have been used for redox-active species loading (Cui et al., 2008; Kong et al., 2012; Gao et al., 2013; Feng et al., 2011; Yang et al., 2014). However, they can hardly be directly used to attach proteins or other biomacromolecules after the upload of signal tags, which is an issue for biosensor fabrication (Wei et al., 2010). Therefore, there is a growing demand to develop a nanomaterial which could easily load redox-active species and proteins, and it becomes the most important thing in fabricating excellent electrochemical immunosensors.

Conjugated polymer–nanoparticle composites, with many advantages such as facile synthesis, substantial solubility, processability, and adjustable moderate conductivity, have received much attention for the design and fabrication of electrochemical immunosensor (Yan et al., 2008; Chen et al., 2008; Devi et al., 2013; Jia et al., 2011; Zang et al., 2013). Generally, there are two main ways to prepare conjugated polymer nanocomposites (CPNC). The first approach is to immobilize nanoparticles on the surface of conjugated polymer through functional groups such as amine or thiols in conjugated polymer via covalent bonds (Pillalamarri et al., 2005; Baker et al., 2011). Whereas, it requires special functional groups for the covalently loading of nanoparticles and limits the application of this method. The second strategy is to modify conjugated polymer with nanoparticles noncovalently via $\pi$–$\pi$ stacking, electrostatic interaction, van der Waals interactions, or hydrogen bonding (Ivanov et al., 2013; Mazeiko et al., 2013). The serious problem associated with the second approach is to remove the residual nanoparticles by tedious washing. To obtain redox-active nanocomposites, the CPNC must load the redox-active species with the same procedure as mentioned above. In summary, the redox-active nanocomposites are usually prepared step by step, which face the the problems of the complicated process and poor stability. Furthermore, its multiple experimental steps and hard-to-control manipulation make it time-consuming and cost-expensive. Thus, it is still a challenge to explore new strategies for further improvement of the simplicity and stability.

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To resolve such problems, here we report a one-step approach to synthesize poly(o-phenylenediamine) (POPD)/Au nanocomposite and poly(vinyl ferrocene-2-aminothiophenol) (poly(VFc-ATP))/Au nanocomposite at room temperature by chemical polymerization. O-phenylenediamine (OPD), a kind of redox-active material, has a planar aromatic structure which can be initiated to be conjugated polymer by radical polymerization when the oxidant exists. Moreover, the amine groups of o-phenylenediamine have the reducing capacity which can be used to prepare metal nanoparticles. Vinyl ferrocene (VFc), an olefin derived from ferrocene, with the excellent redox activity, which can also be polymerized by radical polymerization via initiator. Although it can be used as the redox species, it is hard to be modified with biomolecule or nanoparticles due to the lack of modifiable groups and poor dissolvability. After copolymerize with 2-aminothiophenol (ATP), the amine and thiols groups could be introduced into the chain of the poly(vinyl ferrocene) for improving the biocompatibility and the capacity of loading metal nanoparticles or biomolecules. To initiate the polymerization of OPD, VFc and ATP, $HAuCl_4$ was used as the oxidant, resulting in the formation of POPD/Au and poly(VFc-ATP)/Au nanocomposites, respectively. These conjugated polymer/Au nanocomposites integrated the advantages of excellent redox-activity, facile synthesis, good biocompatibility and stability by one-step method, which have not been reported. The Au nanoparticles (AuNPs) attached densely on the surface of the electroactive polymers in situ by the covalent bonds, which could not only be more stable, but also provide large amount of active sites for immobilizing capture antibodies. Thus, the redox-active polymer/Au nanocomposites were successfully used to fabricate a simple, and sensitive multiplexed electrochemical immunosensor for carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) detection. The electrochemical signals were simultaneously obtained at two peak potentials and the peak currents were dependent on the concentration of the corresponding analytes. The negligible cross-reactivity between the two analytes makes it possesses potential promise in clinical diagnosis.

## 2. Experimental section

### 2.1. Reagents and chemicals

Human immunoglobulin G (IgG), and albumin from bovine serum (BSA) were purchased from Chengwen Biological Company (Beijing, China). Mouse anti human monoclonal antibody to carcinoembryonic antigen (anti-CEA), mouse anti human alpha fetoprotein monoclonal antibody (anti-AFP), CEA and AFP were obtained from Shanghai Linc-Bio Science Co., Ltd. (Shanghai, China). Sodium borohydride ($NaBH_4$, 98%) was purchased from Sigma (USA). Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot XH_2O$), vinyl ferrocene (VFc), D-(+)-glucose, ammonium peroxidisulfate (APS), ascorbic acid (AA), o-phenylenediamine (OPD), uric acid (UA), and 2-aminothiophenol (ATP) were achieved from Alfa Aesar (Tianjin, China). Clinical human serum Samples were obtained from the Capital Normal University Hospital (Beijing, China). $KH_2PO_4$ (99%), HCl, $Na_2HPO_4$ (99%), KCl, potassium ferricyanide ($K_3Fe(CN)_6$), and potassium ferrocyanide ($K_4Fe(CN)_6$) were obtained from Beijing Chemical Reagents Company (Beijing, China). All chemicals were of analytical grade and used without further purification.

### 2.2. Apparatus

The distilled water (resistivity $> 18\ M\Omega$) was used throughout. The morphology of nanocomposites was performed transmission electron microscopy (TEM) with a JEOL-100CX electron microscope under 80 kV accelerating voltage. The elemental analysis was studied by an Escalab 250 X-ray photoelectron spectroscope (Thermofisher, American) employing amonochromatic Al Kα radiation. The structure of the nanocomposites was investigated by Fourier transform infrared spectroscopy (FTIR). All electrochemical measurements were carried out on a CHI-832 electrochemical analyzer (Chenhua, Shanghai, China). A three-electrode electrochemical cell was composed of a modified gold electrode (GE, 4 mm in diameter) as the working electrode, a platinum wire and an Ag/AgCl electrode (saturation of KCl) as the auxiliary electrode and the reference electrode, respectively.

### 2.3. Preparation of POPD/AU nanocomposites

The POPD/Au nanocomposites were synthesized by one-step method. In general, 2.32 mg OPD was added into 4.7 mL of the distilled water with stirring. After the OPD was dissolved, 0.3 mL 4% $HAuCl_4$ was added into the above solution. Then the mixture was vigorously stirred at room temperature for 4 h. The resulting POPD/Au nanocomposites were subsequently centrifuged with ethanol and ultrapure water, and re-dispersed in ultrapure water for use.

### 2.4. Preparation of the poly(VFc-ATP)/AU nanocomposites

The poly(VFc-ATP)/Au nanocomposites were also synthesized by copolymerization in one-step process. Briefly, VFc (0.0212 g) and ATP (2 μL, at room temperature) were mixed in 4.7 mL of distilled water with stirring. After 15 min, 0.3 mL 4% $HAuCl_4$ was added into the above solution and vigorously stirred at room temperature for 4 h. The obtained poly(VFc-ATP)/Au nanocomposites were subsequently centrifuged with ethanol and ultrapure water, and re-dispersed in ultrapure water for use.

### 2.5. Preparation of the poly(2-aminothiophenol) (PATP)

The PATP was synthesized according to our previous work (Liu and Ma, 2014).

### 2.6. Preparation of AuNPs

The AuNPs were synthesized according to the as-reported method (Sun and Ma, 2012). In general, 5 nm AuNPs were prepared at room temperature by adding 1 mL 1% sodium citrate solution to the 100 mL 0.01% $HAuCl_4$ aqueous solution with stirring. After 1 min, 1.6 mL 0.075% $NaBH_4$ (dissolved in 1% sodium citrate solution) was added. The mixture was kept stirring until its color turned to red. The AuNPs were stored at 4 °C for use.

### 2.7. Preparation of immunosensing probes

The immunosensing probes were fabricated by immobilizing labeled anti-CEA and anti-AFP onto POPD/Au and poly(VFc-ATP)/Au nanocomposites, respectively. Firstly, the labeled anti-CEA (100 μL, 1 mg $mL^{-1}$) was mixed with the obtained POPD/Au nanocomposites in 1 mL 0.01 M phosphate buffer (PB, pH 7.3) with gently stirring for 12 h. After centrifugation, the obtained POPD/Au–anti-CEA was incubated in a solution of BSA (10%, w/w) for 2 h to block any possible remaining active sites to avoid any nonspecific absorption. Then, the BSA blocked POPD/Au–anti-CEA was re-dispersed in 1 mL 0.01 M PB (pH = 7.3) and stored in 4 °C for use. The poly(VFc-ATP)/Au nanocomposites were immobilized the labeled anti-AFP with the same procedure as preparing POPD/Au–anti-CEA above. The obtained poly(VFc-ATP)/Au-anti-AFP was re-dispersed in 1 mL 0.01 M PB (pH 7.3) and stored in 4 °C for use.