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Simultaneous electrochemical detection of multiple tumor markers using metal ions tagged immunocolloidal gold

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ABSTRACT

In this work, a sandwich-format electrochemical immunosensor has been fabricated in the aim of simultaneous detection of carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) using metal ions tagged immunocolloidal gold nanocomposites as signal tags. The capture anti-CEA and anti-AFP were immobilized onto the chitosan–Au nanoparticles (CHIT–AuNPs) membrane modified glassy carbon electrode through glutaraldehyde (GA). The metal ion labels could be detected directly through differential pulse voltammetry (DPV) without metal pre-concentration, and the distinct voltammetric peaks had a close relationship with each sandwich-type immunoreaction. Under the optimized conditions, the multiplexed immunoassay exhibited good sensitivity and selectivity for the simultaneous determination of CEA and AFP with linear ranges of 0.01–50 ng mL^{-1}. The detection limits for CEA and AFP are 4.6 pg mL^{-1} and 3.1 pg mL^{-1}, respectively. The method was successfully applied for the determination of AFP and CEA levels in clinical serum samples, and the results were in good agreement with standard enzyme linked immunosorbent assay (ELISA). This approach gives a promising simple and sensitive immunoassay strategy for clinical applications.

1. Introduction

Early and sensitive determination of tumor markers played a crucial role in clinical diagnosis (Lefkowitz et al., 2010) since the precise and early diagnosis of tumor markers could greatly improve the treatment efficiency of many cancers. The detection of single protein has been well developed, however, the diagnostic value of a single tumor marker determination is often limited because most markers are not specific to a particular tumor (Wu et al., 2008; Tang et al., 2011). Therefore, the investigation in simultaneous determination of two or more tumor markers has become popular in clinical laboratories as it could provide a more reliable measurement (Jia et al., 2014). Meanwhile, the multiplexed immunoassay which quantitatively detects a panel of biomarkers can greatly improve the diagnostic specificity. Various immunoassay methods for simultaneous detection have been developed in recent years such as Raman spectroscopy, fluorescence, chemiluminescence, surface plasmon resonance (Xu et al., 2011; Song et al., 2009; Olsen et al., 2006; Bisoffi et al., 2008). Particularly, electrochemical immunoassays attracted extensive interest for the advantages of portability, low cost and high sensitivity (Chikkaveeraiah et al., 2012), which makes it an ideal strategy.

Searching distinguishable signal tags to load electroactive species as trace labels for tumor markers has always been the first issue to achieve simultaneous multianalytes determination in electrochemical immunoassays. The traditional signal tags such as toluidine blue, Prussian blue, ferrocene, thionine have been widely used and reported (Han et al., 2012; Bai et al., 2012; Yang et al., 2012; Liu and Ma, 2013). In order to get a higher sensitivity, however, it often needs signal amplification strategy such as enzymatic reaction, making the operation process much more complex. Metal nanoparticle or quantum dots can show distinct inerratic voltammetric peaks without any signal amplification strategy, have the potential to work as ideal redox probe, but a complicated
and time-consuming acid dissolution step and metal pre-concentration is commonly indispensable to get metal ions before electrochemical detection (Wang et al., 2003). Recently, Feng et al. (2012) developed titanium phosphate nanospheres with excellent ion-exchange property which could directly immobilize metal ions Zn^{2+} and Cd^{2+} on the surface. However, since the fabrication of template for metal ion exchange is another time-consuming job, there still exists challenge to explore new strategies for immobilizing metal ions on probes.

Nanostructures with amino-groups showed good adsorption properties for Pd^{2+}, Ag^{+}, Ni^{2+}, Cu^{2+}, Co^{2+}, and Cd^{2+} (Yi et al.,

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2006), therefore, it provided another more simple strategy for immobilizing metal ions on immunosensing probes. In this work, the immunocolloidal gold came from gold nanoparticles (AuNPs) conjugated with antibody (anti-CEA, anti-AFP) was prepared for simultaneous detection of CEA and AFP. The Cu^{2+} and Pb^{2+} were used as electrochemical tags for their different redox potential and strong binding capacity with amino-groups of protein. In addition, the voltammetric peaks of Cu^{2+} and Pb^{2+} are near 0.05 V and −0.5 V, respectively. These two voltammetric peaks were apparent and not far separated, reducing the time needed for DPV test. For the immunosensing platform, we chose chitosan–Au nanoparticles (CHIT–AuNPs) as layer to immobilize capture antibody. CHIT has a good biocompatibility, excellent film-forming ability, strong adhesion and high mechanical strength, which make it an ideal matrix for biomarcromolecules immobilization (Sheng et al., 2008). The AuNPs packaged in the membrane act as an excellent nanomaterial with good conductivity can greatly promote electron transfer between the probes and electrode surface (Sun and Ma, 2012 Liu and Ma, 2014). To further simplify the procedure, the AuNPs–CHIT was fabricated by using the one step heating method without adding any other reductant, which is very different from our previous work (Chen et al., 2013). Compared with the routine method, present work shows three advantages: (1) the loading process of metal ions on immune probes and the fabrication of nanocomposites were greatly simplified; (2) no enzymatic reaction is needed to enhance the signal but still obtain a well sensitivity; (3) the metal ion label could be detected directly through differential pulse voltammetry (DPV) without metal dissolution and pre-concentration steps.

In this proposed immunosensor, the simultaneous determination of multianalytes has been conducted on one electrode through these multiple labels. For the detection of clinical serum samples, the result was well consistent with that determined by the ELISA. The linear range (0.01 ng mL^{-1} to 50 ng mL^{-1}) completely meet the requirements of clinical diagnosis for threshold values in normal human serum is ~3 ng mL^{-1} for CEA and 10 ng mL^{-1} for AFP (Wu et al., 2007), indicating that the present immunosensor provides a possible application for the simultaneous determination of CEA and AFP in clinical diagnostics.

2. Experimental section

2.1. Reagents and materials

CEA was purchased from Biosynthesis Biotechnology Company (Beijing, China). Alpha-fetoprotein (AFP), monoclonal anti-CEA and anti-AFP capture antibodies, monoclonal anti-CEA and anti-AFP labeled antibodies were purchased from Linc-Bio Company (Shanghai, China). Hydrogen tetrachloroaurate hydrate ($HAuCl_4 \cdot xH_2O$, 99.9%) was purchased from Alfa Aesar China (Tianjin). Sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich (USA). Chitosan (CHIT) was obtained from TCI (Tokyo, Japan). NaH_2PO_4, Na_2HPO_4, K_2CO_3, $Cu(NO_3)_2$, $Pb(NO_3)_2$ and bovine serum albumin (BSA) were purchased from Beijing Chemical Reagents Company (Beijing, China). Clinical serum samples were provided by Hospital of Capital Normal University, China. And ultrapure water (resistivity > 18 MΩ) was used throughout the experiment. All the reagents were of analytical grade and used as received.

2.2. Apparatus

In all the procedures, the water used was purified through an Olst ultrapure K8 apparatus (Olst, Ltd., resistivity > 18 MΩ). Transmission electron micrographs (TEM) of the nanomaterials were taken with a Hitachi (H7650, 80 kV) transmission electron microscope. X-ray photoelectron spectroscopy (XPS) analysis was performed on an Escalab 250 X-ray Photoelectron Spectroscope (Thermofisher, American) using an Al (mono) Kα radiation. All electrochemical experiments were carried out on a CHI1140 electrochemical workstation (Chenhua Instruments Co., Shanghai, China). A three-electrode system consist of a platinum wire as the auxiliary electrode, an Ag/AgCl electrode (saturated KCl) as the reference electrode and a glassy carbon electrode (GCE) (4 mm in diameter) as the working electrode was used in the experiment.

2.3. Preparation of CHIT–AuNPs nanocomposites

The CHIT–AuNPs was synthesized according to the previous protocol with a little modification (Huang et al., 2004). Briefly, 100 μL 1% $HAuCl_4$ aqueous solution was mixed with 10 ml 0.2 (w/w)% chitosan (in 1% acetic acid aqueous solution). The mixture was heated to reflux for 1 h while stirring. After cooling down to room temperature, the obtained nanocomposite was kept at 4 °C for further use. And the size of AuNPs packaged in chitosan was about 20–50 nm.

2.4. Preparation and modification of AuNPs

Big AuNPs (55 nm or bigger) was less stable in solution than small AuNPs (such as 20 nm) and the aggregation occurs in the storage process. Thus, 20 nm AuNPs were chosen for further experiments. The AuNPs with sizes of 20 nm used in this work were prepared according to our previous work (Wang et al., 2006). Four milliliters 1% sodium citrate solution was added to 100 mL 0.01% boiling $HAuCl_4$ solution with vigorous stirring. The color changed from pale to blue, then to burgundy. Boiling was continued for 15 min and then stirring until the sample had cooled to room temperature.

2.5. Preparation of the immunosensing probes

Under gently stirring, 0.2 M K_2CO_3 aqueous solution was added drop by drop to 2 ml colloidal gold as prepared until the pH to 9. Then labeled anti-CEA (200 μL, 1 mg mL^{-1}) was added to the dispersion and stirring at room temperature for 2 h. After centrifugation, 200 μL 2% BSA solution was added to the obtained bioconjugates and allowed to react for 2 h. Then the remaining active sites were blocked and the non-specific adsorption was eliminated. The resulting nanocomposite was then collected by centrifugation at 15,000 rpm for 10 min and washed three times with ultrapure water. Then, the prepared nanocomposite was dispersed in 2 mL 10 mM $Cu(NO_3)_2$ aqueous solution and reacted for 12 h under gently stirring. AuNPs conjugated with labeled anti-AFP was prepared in the same way, and the resulting nanocomposite was dispersed in 2 mL 10 mM $Pb(NO_3)_2$ aqueous solution and reacted for 12 h under gently stirring. After centrifuged and washed for several times, the obtained bioconjugates were re-dispersed in 2 mL ultrapure water and stored at 4 °C for further use.

2.6. Fabrication of the immunosensor

The GCEs decorated with capture anti-CEA and capture anti-AFP was prepared through the following approach: GCE (4 mm in diameter) was carefully polished with 1.0, 0.3 and 0.05 μm alumina powder separately, and rinsed thoroughly with bi-distilled water between each polishing step. Followed by successive sonication in double distilled water and ethanol for 5 min and dried in nitrogen, 10 μL CHIT–AuNPs solution was cast on to the electrode carefully and dried at 30 °C for 30 min. Then a