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Conductive carbon nanoparticles-based electrochemical immunosensor with enhanced sensitivity for α-fetoprotein using irregular-shaped gold nanoparticles-labeled enzyme-linked antibodies as signal improvement

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ABSTRACT

A new electrochemical immunoassay protocol for sensitive detection of α-fetoprotein (AFP, as a model) is designed using carbon nanoparticles (CNPs)-functionalized biomimetic interface as immunosensing probe and irregular-shaped gold nanoparticles (ISNGs)-labeled horseradish peroxidase-*anti*-AFP conjugates (HRP-*anti*-AFP-ISNG) as trace label. The low-toxic and high-conductive CNPs provided a high capacity nanoparticulate immobilization surface and a facile pathway for electron transfer. In comparison with conventional label methods, i.e. spherical gold nanoparticles-labeled HRP-*anti*-AFP and HRP-labeled *anti*-AFP, the electrochemical immunosensor using HRP-*anti*-AFP-ISNGs as trace labels exhibited high bioelectrocatalytic response toward enzyme substrate and a wide dynamic range from 0.02 to 4.0 ng/mL with a low detection limit of 10 pg/mL toward AFP (at 3σ). The developed immunoassay method showed good selectivity and acceptable reproducibility. Clinical serum samples with various AFP concentrations were evaluated by using the electrochemical immunosensor and the referenced enzyme-linked immunosorbent assay (ELISA), respectively, and received in good accordance with results obtained from these two methods.

1. Introduction

Accurate and sensitive determination of disease-related proteins is very important in many modern research fields including biochemistry, biomedicine, and diagnostic (Lefkowitz et al., 2010; Dutsch-Wicherek, 2010). Especially, the clinical measurement of tumor-related proteins and infectious disease-related proteins plays a greatest interest in early disease detection and highly reliable predictions (Gunaseelan et al., 2010; Coyle and Johnston, 2010). Various immunoassay methods have been reported for the detection of tumor markers, such as electrochemical enzyme immunoassay (Martinez et al., 2010; Chikkaveeraiah et al., 2009), fluorescence immunoassay (Zheng and Li, 2010; Liu et al., 2010), chemiluminescence immunoassay (Yang et al., 2009), enzyme-linked immunosorbent assay (Zhou et al., 2010), radioimmunoassay, and as on. Despite many advances in this field, it is still a challenge to find new approaches that could improve the simplicity, selectivity, and sensitivity of clinical immunoassay.

Nanobiotechnology has experienced tremendous growth on the successful development of new analytical tools and instrumentation for bioanalytical and biotechnological applications (Feldmann and Goesmann, 2010; Verma and Stellacci, 2010). The application of these nanobiotechnology in biosensing in place of conventional sensing technologies has led to improvements in sensitivity, selectivity, and multiplexing capacity (Floss et al., 2010; Sanvicens et al., 2009). Carbon nanomaterials with extraordinary physical properties and remarkable conductivities have attracted considerable attention for the successful development of electrochemical sensors, and it mainly includes carbon nanotube, carbon nanoparticles and graphene (Rao et al., 2009; Capek, 2009). Carbon nanotubes have extensively used as an immobilized substrate for the fabrication of biosensor (Tran et al., 2009). The three-dimensional nanostructures enhanced the immobilized amount of biomolecules. Recently, Ju and co-authors reported an ultrasensitive electrochemical immunosensor using carbon nanotube as labels (Zhang et al., 2010). However, a major limitation of using carbon nanotubes is to improve the heterogeneity and purity of carbon nanotubes because they were usually dispersed using other solvents during the experimental process. Graphene with high electrochemical activity was also used for the construction of biosensor and bionanolabels (Drissi et al., 2010; Dong and Chen, 2010; Geim, 2009). One of the problems commonly associated with graphene is to decrease the surface coverage of the electrode due to its two-dimensional network. Carbon nanoparticles (CNPs) have been of considerable research attention due to their intrinsic chemical

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(sp^2-π electrons) and physical (amorphous carbon nanoparticles) properties (Victor et al., 2006), and they are different structures comparing to fullerences or carbon nanoonions, because they have unclosed graphene layers. In addition, one major advantage using CNPs is to be easily prepared and exhibit field emission properties comparable to carbon nanotubes due to the high degree of graphitization (Yu et al., 2001). Another issue is that CNPs possessed unique properties including low density, high porosity and surface area, and relatively high chemical and thermal stability (Panessa-Warren et al., 2006). In these regards, CNPs could find possible prospective applications, e.g. as catalysts supports, lubricaing agents, electrode materials and in construction of advanced sensors. In this study, we used the carbon nanoparticles with low toxicity and high conductivity as the immobilized substrate for the conjugation of biomolecules.

For the successful development of sandwich-type electrochemical immunoassays, the label of detection antibodies (i.e. secondary antibodies) is critical. Usually, bioactive enzyme (e.g. horseradish peroxidase) molecules were conjugated to the secondary antibodies. However, the labeled amount of enzyme is limited on each antibody. Recently, various labeled methods have been developed to enhance the sensitivity (Liu and Lin, 2007). Gold nanoparticle label with easy preparation and good biocompatibility has been extensively employed to label different biological receptors, including antibody and enzyme (Ambrosi et al., 2007). Except for gold colloids, carbon nanotubes/nanospheres and nanosilica have already used for the label of secondary antibodies (Zhang et al., 2010; Du et al., 2010). In our previous work, we also developed several novel sandwich-type electrochemical immunoassay protocols for the detection of tumor markers using magnetic nanogold microspheres (Tang et al., 2008), nanogold hollow microspheres (Tang and Ren, 2008), and magnetic nanosilica microspheres (Tang et al., 2010) as label probes. However, we recently found that gold nanoparticles with various shapes displayed different electrochemical characteristics when they were used as the label probes.

The aim of this work is to probe into the effect of gold nanoparticle-labeled probe with irregular shape on the properties of the electrochemical immunosensors using carbon nanoparticles-functionalized immunosensing interface (AFP, as a model tumor marker). The immunosensor was fabricated using *anti*-AFP/chitosan/CNPs-modified screen-printed carbon electrode. The irregular-shaped gold nanoparticles with a mean length of ~50 nm and a narrowing width from 25 to 30 nm along its longitudinal axids were used for the label of secondary antibodies. The new nanolabels were expected to improve the electrochemical signal of the sandwich-type electrochemical immunoassays in this study.

2. Experimental

2.1. Materials and reagents

Monoclonal *anti*-AFP antibody produced in mouse (clone 1G7, buffered aqueous solution), AFP from human fetus, and HRP-*anti*-AFP purchased from Zhengzhou Biocell Biochem. Co. (Zhengzhou, China). Carbon nanoparticles (CNPs; 14 nm in diameter; Printex 90) was obtained from Degussa (Frankfurt, Germany). Chitosan from crab shells (<200 mPa s, 1% in acetic acid, 20 °C), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and bovine serum albumin (BSA) were achieved from Sigma–Aldrich (St. Louis, MO, USA). Hydrogen tetrachloroaurate (III) tetrahydrate ($HAuCl_4{\cdot}4H_2O$) was purchased from Sinopharm Group Chem. Re. Co. Ltd. (Changhai, China). All other reagents were of analytical grade and were used without further purification. Deionized and distilled water was used throughout the study. Phosphate-buffered saline (PBS, 0.1 M) of various pHs were prepared by mixing of the stock solutions of Na_2HPO_4 and NaH_2PO_4. Stock suspensions (1.0 mg/mL) of carbon particles were prepared in pH 7.4 PBS by sonication for 60 min at 50–60 Hz, 120 W. Graphite powder (no. 50870) was from Fluka. The solid paraffin substrates for screen-printed electrodes were from Chongqing Chem. Ltd. Co. Silver-based ink was from GEM-Gwent (Pontypool, UK).

2.2. Synthesis and bioconjugation of irregular-shaped gold nanoparticles (ISNG)

Prior to experiment, we synthesized the irregular-shaped gold nanoparticles (ISNGs) consulting the literature (Wang et al., 2006) as follows: (i) 50 mL $HAuCl_4{\cdot}4H_2O$ (0.1 mM) aqueous solution and 5 mL starch (0.17 wt.%) were initially added into a 150-mL round-bottom flask in turn, and purged for 10 min by oxygen with argon; (ii) 1.0 mL D-glucose (1.0 mM) purged with argon was introduced to the mixture, and stirred with 750 rpm for 24 at 40 °C until the mixture turned light violet. The obtained ISNGs were characterized by transmission electron microscopy (TEM, H600, Hitachi Instrument, Japan) and UV–vis spectra (Tianmei UV 1102, China).

The bioconjugation of the synthesized ISNGs (HRP-*anti*-AFP-ISNGs) was prepared according to the literature (Li et al., 2009): 20 mL colloidal ISNG solution was initially adjusted to pH 9.0–9.5 using Na_2CO_3, and then 1.0 mL of the original HRP-*anti*-AFP was added into ISNG colloids. After incubated for 12 h at 4 °C with slight stirring, the mixture was centrifugated (13,000 rpm) at room temperature (RT) for 30 min. The obtained pellet was resuspended into 2.0 mL pH 7.4 PBS containing 1.0 wt.% BSA. The synthesized bionanolabels were stored at 4 °C when not in use. For comparison, the 31-nm spherical gold nanoparticles (NG) were prepared according to the literature (Frens, 1973), and labeled with HRP-*anti*-AFP molecules (HRP-*anti*-AFP-NGs) using the same method.

2.3. Fabrication of the electrochemical immunosensor

The electrochemical immunosensor was fabricated on a screen-printed carbon electrode (SPCE), which included a 2-mm graphite working electrode, a Ag/AgCl reference electrode and a graphite auxiliary electrode. A DEK 1202 semi-automatic screen-printing machine with a 156 threads/in. polyester screen, a stainless steel flood blade, and a polyurethane squeegee (Weymouth, UK) was used for the fabrication of SPCE. The counter electrode and the conducting track of working electrode were prepared from the graphite-CA ink *via* mixing 1.4 g of graphite powder in CA binder already dissolved in cyclohexanone (3.4 g of solution at 7%, w/w). The thickness of printed layer was about 0.5 mm. The insulating layer printed around the working area constituted an electrochemical detection cell.

The electrochemical immunosensor were fabricated as the following steps: 0.1 mg carbon nanoparticles was initially added to 1.0 mL chitosan acetic acid solution (0.2 wt.%, pH 5.0), and continuously sonicated for 30 min to achieve a homogeneous suspension. Following that, 5 μL suspension was cast on the surface of the cleaned carbon working electrode, and dried for 2 h at room temperature. Afterward, 10 μL mixture solution containing 400 mM EDC and 100 mM NHS was dropped on the surface of CNP-chitosan/SPCE, and incubated for 30 min at room temperature to activate the $-NH_2$ groups of chitosan. After washed with pH 7.4 PBS, 10 μL *anti*-AFP primary antibodies (0.5 mg/mL) were quickly thrown the surface, and incubated for 3 h at 37 °C. Subsequently, the resulting SPCE was washed 3 times with pH 7.4 M PBS containing 0.05% (w/v) Tween 20 to remove the physically absorbed biomolecules. The structure and functionalization of the SPCE are shown in Fig. 1.