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Simultaneous detection of two tumor markers using silver and gold nanoparticles decorated carbon nanospheres as labels

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ABSTRACT

In this work, a multiplexed electrochemical immunosensor was developed for sensitive detection of carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) using silver nanoparticles (Ag NPs) or gold nanoparticles (Au NPs) coated-carbon nanospheres (CNSs) as labels. CNSs were employed as the carrier for the immobilization of nanoparticles (Ag NPs or Au NPs), thionine (Thi), and secondary antibodies (Ab_2) due to their good monodispersity and uniform structure. Au NPs reduced graphene oxide (rGO) nanocomposites were used as sensing substrate for assembling two primary antibodies (Ab_1). In the presence of target proteins, two labels were attached onto the surface of the rGO/Au NPs nanocomposites via a sandwich immunoreaction. Two distinguishable peaks, one at +0.16 V (corresponding to Ag NPs) and another at −0.33 V (corresponding to Thi), were obtained in differential pulse voltammetry (DPV). The peak difference was approximately 490 mV, indicating that CEA and AFP can be simultaneously detected in a single run. Under optimal conditions, the peak currents were linearly related to the concentrations of CEA or AFP in the range of 0.01–80 ng ml^{-1}. The detection limits of CEA and AFP were 2.8 and 3.5 pg ml^{-1}, respectively (at a signal-to-noise ratio of 3). Moreover, when the immunosensor was applied to serum samples, the results obtained were in agreement with those of the reference method, indicating that the immunosensor would be promising in the application of clinical diagnosis and screening of biomarkers.

Recently, the multiplexed electrochemical immunosensor for the detection of multiple tumor markers has caused considerable attention because it can provide useful and reliable information for clinical diagnosis [1–3]. As is well known, carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) are two common tumor markers, and the content of them in human serum is related to colorectal cancer, breast cancer, and liver cancer [4]. Therefore, it is necessary to develop sensitive multiplexed electrochemical immunosensors for detection of CEA and AFP [5–8]. For example, Niu's group [9] used ITO (indium tin oxide) electrode modified with gold nanoparticles and mesoporous silica to develop an immunosensor for simultaneous detection of CEA and AFP. The immunosensor exhibited wide linear response ranges for CEA (0.5–50 ng ml^{-1}) and AFP (0.5–100 ng ml^{-1}). The detection limit was 0.1 ng ml^{-1} in both cases. Ma's group [10] employed chitosan–gold nanoparticles modified electrode to develop a multiplexed immunosensor for the detection of CEA and AFP. The linear response range for both analytes was within 0.5–60 ng ml^{-1}. The detection limits were 0.1 ng ml^{-1} for CEA and 0.05 ng ml^{-1} for AFP.

From principle, there are several key factors for influencing the performance of multiplexed immunosensor. One of them is to search suitable nanomaterials with abundant activity sites to immobilize signal probes and antibodies such as carbon nanomaterials [11,12], silica nanoparticles [13], and magnetic nanoparticles [14]. For example, Liu's group [15] adopted silica nanoparticles to immobilize quantum dots as labels for

Abbreviations used: CEA, carcinoembryonic antigen; AFP, α-fetoprotein; CNSs, carbon nanospheres; Thi, thionine; NP, nanoparticle; Ag, silver; Ab_2, secondary antibodies; Au, gold; rGO, reduced graphene oxide; Ab_1, primary antibodies; DPV, differential pulse voltammetry; PSA, prostate-specific antigen; BSA, bovine serum albumin; PDDA, poly(diallyldimethylammonium chloride); EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffer solution; SCE, saturated calomel electrode; GCE, glassy carbon electrode; EIS, electrochemical impedance spectroscopy; SEM, scanning electron microscopy; CS, chitosan; UV–vis, ultraviolet–visible; CV, cyclic voltammetry; Glu, glucose; AA, ascorbic acid; ELISA, enzyme-linked immunosorbent assay.

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simultaneous electrochemical detection of immunoglobulin G (IgG) antigen and CEA. Wei's group [16] employed magnetic nanoparticles to immobilize thionine and ferrocenecarboxylic acid and realized simultaneous detection of squamous cell carcinoma associated antigen and CEA. In those works, silica nanoparticles and magnetic nanoparticles provided larger surface area for immobilizing amounts of signal molecules and antibodies. As a result, these immunosensors exhibited high sensitivity. However, silica nanoparticles and magnetic nanoparticles suffered from poor water solubility and complicated synthesis, and the two nanoparticles were limited in the electrochemical immunosensor field. Carbon nanospheres (CNSs) possess some advantages such as excellent biocompatibility, and their size, shape, and surface properties can be finely tuned via controlling experiment parameters [17,18]. More important, the CNSs show good adsorption capacity to signal probes (e.g., organic dyes, quantum dots [19]). Hence, CNSs have been become an ideal material for the immobilization of signal probes and antibodies.

Another important factor is to search signal molecules that possess distinguishable responses to each other. Signal molecules may be organic dyes (e.g., thionine, Thi), nanoparticles (NPs; e.g., PbS NPs, Ni NPs, silver [Ag] NPs), or other redox molecules (e.g., ferrocene) [20–23]. Based on the above considerations, in the current work we selected Ag NPs and Thi as signal molecules, and CNSs were employed for the immobilization matrix of signal molecules and secondary antibodies (Ab_2). Gold NPs reduced graphene oxide nanocomposites (rGO/Au NPs) were used as sensing substrate for assembling two primary antibodies (Ab_1). In the presence of two analytes, the "sandwich"-type immunocomplex was formed via specific interaction of antibody–antigen. Differential pulse voltammetry (DPV) was employed to record the sensing signals. Two distinguishable peaks, one at +0.16 V (corresponding to Ag NPs) and another at −0.33 V (corresponding to Thi), were obtained. The peak potential difference was approximately 490 mV, indicating that CEA and AFP can be detected simultaneously in a single run. The experimental results showed that the obtained immunosensor exhibited good selectivity and high sensitivity, which can be applied to multiplexed analysis of tumor markers in real samples. In contrast to the earlier similar work, the immunosensor showed some advantages. First, the immunosensor does not require harsh detection conditions such as an acid dissolution step [15] and deoxygenation [24]. Second, no enzymatic reaction is needed to enhance the signal [25]. Third, the as-prepared nanocomposites, Ag NPs@CNSs and Au NPs–Thi@CNSs, provide large surface area and active sites to immobilize amounts of second antibodies that greatly improve the signal intensity.

Materials and methods

Reagents

CEA, anti-CEA antibody, AFP, anti-AFP antibody, and prostate-specific antigen (PSA) were purchased from Biocell Biotech (Zhengzhou, China). Bovine serum albumin (BSA) was purchased from Sinopharm Chemical Reagent (Shanghai, China). rGO (purity $\geq$ 98%) was purchased from Sinocarbon Materials Technology (Taiyuan, China). Poly(diallyldimethylammonium chloride) (PDDA, average MW = 100,000–200,000, low molecular weight, 20 wt%) and Thi were obtained from Aldrich (St. Louis, MO, USA). Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), Silver nitrate ($AgNO_3$), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Shanghai Chemical Reagent (Shanghai, China). Phosphate buffer solution (PBS) was prepared with NaH_2PO_4 and Na_2HPO_4. Blocking solution was 1% BSA. Twice-distilled water was used in the study. The serum samples were provided by the clinical laboratory of the Yiji Shan Hospital (Wuhu, China) with informed consent. All experiments were performed in compliance with the relevant laws and institutional guidelines of the ethics committee.

Apparatus

All electrochemical measurements were performed on a CHI650C electrochemical analyzer (CH Instruments, China). A conventional three-electrode system was employed with a platinum electrode as auxiliary, a saturated calomel electrode (SCE) as reference, and a bare glassy carbon electrode (GCE) or modified GCE as working electrode.

Electrochemical impedance spectroscopy (EIS) was performed in 0.1 M PBS containing 5.0 mM $[Fe(CN)_6^{3-/4-}]$ and 0.1 M KCl. The frequency range was from 0.1 to 100 kHz, and the amplitude of the alternate voltage was 5 mV. Morphologies of various nanomaterials and nanocomposites were obtained with scanning electron microscopy (SEM; S-4800, Hitachi, Japan). An energy dispersive X-ray spectrometer (EDS) was employed for nanomaterials analysis.

Preparation of CNSs@Ag NPs and CNSs@Thi–Au NPs

CNSs were first prepared according to the literature [26,27]. Briefly, 4 g of glucose was dissolved in 40 ml of ultrapure water and placed in a 50 ml Teflon-sealed autoclave, which was maintained at 180 °C for 6 h. The black suspension obtained was isolated by centrifugation. CNSs were obtained by repeatedly washing with ethanol and water and drying under vacuum at 60 °C overnight. To generate carboxylic groups on the surfaces of CNSs, the obtained CNSs were treated with a mixture acid of H_2SO_4, HNO_3, and ultrapure water (3:1:6, v/v) for 2 h under stirring. Thus, the surface of CNSs was functionalized with −COOH. The resulting dispersion was treated by centrifugation and washing, and the carboxylated CNSs obtained were dried at 80 °C for 12 h.

The Ag NPs coated CNSs (CNSs@Ag NPs) were prepared as follows. First, 15 mg of CNSs was dispersed in 20 ml of 0.25% PDDA solution containing 20 mM NaCl and stirred gently for 30 min to give a homogeneous brown suspension, and then the suspension was washed and dried at 40 °C. Second, 10 mg of PDDA-functionalized CNSs was dispersed in 10 ml of Ag NPs colloid solution under ultrasonic stirring. During this process, Ag NPs were assembled on the surface of CNSs via electrostatic interaction. After centrifugation and washing, the precipitates were redispersed in 2.0 ml of PBS (pH 7.0) for further use.

The preparation of Au NPs coated CNSs–Thi nanocomposites (CNSs@Thi–Au NPs) consisted of two steps. First, 100 μl of 10 mg ml^{-1} EDC and 100 μl of 10 mg ml^{-1} NHS were injected into 2.0 ml of 0.5 mg ml^{-1} carboxylated CNSs. After 30 min, 2.0 ml of 1.0 mM Thi was added into the solution above and stirred vigorously for 12 h. During this process, Thi was covalently bound to the surface of CNSs via amidation reaction. The obtained CNSs@Thi suspension was used for the next step. Second, the CNSs@Thi was added dropwise into 10.0 ml of Au NPs under gentle stirring. During the process, Au NPs were assembled on the surface of CNSs@Thi via the Au–NH bond. After that, The mixture was centrifugal treated, and the precipitate was redispersed in 2.0 ml of PBS (pH 7.0).

Preparation of two labels

First, 100 μl of 1 mg ml^{-1} anti-CEA (Ab_2) was injected into the CNSs@Ag NPs suspension and stirred gently for 12 h, and the free antibodies were removed through centrifugation and washing. The obtained CNSs@Ag NPs–Ab_2 bioconjugates were dispersed in 1% BSA solution to block out active sites. After centrifugation and