Sandwich-type Electrochemical Immunoassay for Carbohydrate Antigen-125 Using Multifunctional Magnetic Beads with Ferrocenyl-tethered Dendrimer as Label

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This letter describes a new sandwich-type electrochemical immunoassay for carbohydrate antigen-125 (CA125) by using multifunctional magnetic beads with ferrocenyl-tethered dendrimer as labels.

Amplified electrochemical signals can significantly enhance the sensitivity of electrochemical immunoassays, but often require expensive and complex detectors, which limit their broad use. Some promising approaches have been employed for signal amplification, such as enzymes,[1] nanoparticles,[2] DNA-based polymerase chain reaction (PCR),[3] and liposome-PCR assay.[4] Recently, the biobarcode amplification assay has become a powerful tool in detecting tens to hundreds of biological targets such as proteins and nucleic acids in an entire sample, which combines tunable nanoparticle features with the unique physical and chemical characteristics of protein and peptides. Mirkin's group utilized oligonucleotides as biochemical barcodes for detecting multiple protein structure in one solution.[5,6] The hybridization events that resulted in the aggregation of gold nanoparticles significantly altered their physical properties. Merkoci et al. reported a novel double-codified nanolabel AuNPs-labeled anti-human IgG antibody for the determination of IgG.[7]

Gold nanoparticles with high surface-to-volume ratio and good biocompatibility have been used for the immobilization of antibodies in the previous report.[8] Recently, Tang and coworkers reported two novel electrochemical sandwich-type immunoassays of biomarkers by using thionine-doped magnetic gold nanospheres and horseradish peroxidase-encapsulated nanogold hollow microspheres as labels, respectively.[1,9] To date, however, there are few reports focusing on electrochemical studies for the antigen–antibody reactions using multifunctional magnetic nanoparticles (MMBs) as labels.

CA125 is a membrane mucin-like glycoprotein greater than 200 kDa with a threshold value of 35 U mL^{-1}, high levels of which have been found in ovarian cancer and has been used for monitoring the course of epithelial ovarian tumors. Herein, a redox-active MMBs was initially synthesized by using magnetic Fe_3O_4 nanoparticles as core and partially ferrocenyl-tethered dendrimer (Fc-D, Sigma) as shell, and then the prepared MMBs were used as an affinity support for the conjugation of anti-CA125 antibodies (Figure 1a). Dendrimers are unique monodisperse polymers that have a globular shape and branched structure, and molecular size and the number of surface functional groups are controllable during synthesis. With a sandwich-type immunoassay format, immunocomposites were formed by an anti-CA125-modified glassy carbon electrode (GCE), CA125 in the sample solution and anti-CA125-coated MMBs. Due to the presence of Fc-D, the peak current of the modified GCE increased with the increment of CA125 concentration in the sample. The detection process is illustrated in Figure 1b.

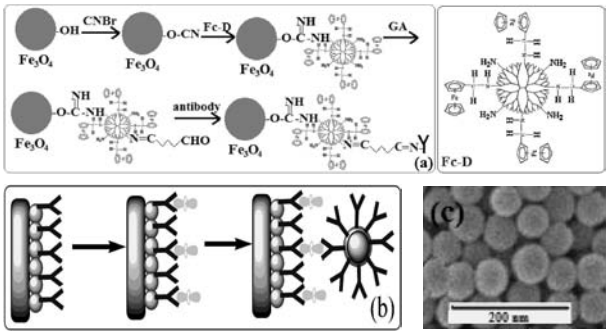

Figure 1. Fabrication process of (a) the MMBs, (b) the electrochemical immunoassay, and (c) SEM image of the synthesized MMBs.

Prior to experiments, 24-nm Fe_3O_4 nanoparticles were synthesized according to the literature.[10] The synthesis of MMBs was performed as follows (Figure 1a): 0.2-g Fe_3O_4 nanoparticles were added to a CNBr solution (2.0 g mL^{-1} in acetone) for 5 min at room temperature. Prepared in this way, the surface of the magnetic nanoparticles was exposed to active –OCN groups that could react readily with the $–NH_2$ groups. After washing with ice cold water for 4 times, the activated nanoparticles were dispersed into partially ferrocenyl-tethered dendrimer (Fc-D) aqueous solution, and stirred for 6 h at room temperature under a nitrogen atmosphere (Notes: The excess Fc-D was removed by magnetic separation). After washing 3 times with 0.05 M HCl and ultra-pure water alternately, the Fc-D–Fe_3O_4 nanoparticles were reacted with 2.5% glutaraldehyde (GA) (pH 3.5) at room temperature for 6 h. The products were enriched with the aid of an external magnet. Following that, 300 mL of 400 U mL^{-1} anti-CA125 was added to the GA/Fc-D/Fe_3O_4 nanocomposites, and incubated for 12 h at 4 °C with frequent stirring. Finally, the formed MMBs were obtained by magnetic separation, and stored at 4 °C when not in use.

To comprehend the size of the synthesized MMBs, we used scanning electron microscopy (SEM, Jeol Ltd., Tokyo, Japan) to characterize various magnetic beads. The mean sizes were 24 and 35 nm for Fe_3O_4 nanoparticles and MMBs, respectively. Moreover, there are some flagellum-like structures around the synthesized MMBs (Figure 1c). To further investigate whether the Fc-D has been conjugated on the Fe_3O_4 nanoparticles, we used atomic emission spectrometry (Puxin, Beijing, China) to quantitatively calculate the mass of Fe at 283.204 nm before and after modification of Fc-D. Seen from experimental data, the contents of Fe were 0.1514 and 0.1978 g for Fe_3O_4 nanoparticles and MMBs, respectively. The mass increment of Fe element suggested that the Fc-D was bound onto the MMBs.

To prepare a sandwich-type heterogeneous electrochemical immunosensor, a simple method was adopted. Initially, gold

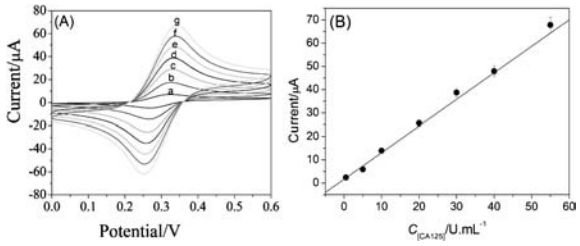

Figure 2. (A) Cyclic voltammograms of the formed sandwich-type immunosensor toward various concentrations of CA125 in pH 7.0 PBS, a–g; 0.5, 5, 10, 20, 30, 40, and 55 U mL^{-1}, respectively), (B) calibration curves for the electrochemical sandwich-type immunoassay of CA125.

nanoparticles were electrochemically deposited on a cleaned GCE by potential-step electrolysis from +1.1 to 0 V in 0.5 M H_2SO_4 solution containing 1.0 mM $HAuCl_4$ with different pulse time, i.e., 10, 30, and 60 s.[9] Then, the nanogold-modified GCE was dipped into 400 U mL^{-1} anti-CA125 solution for 6 h at 4 °C. The resulting GCE was used as the base electrode for the detection of CA125. First, we investigated the amperometric responses of the sandwich-type immunoassay by using the MMBs as labels in pH 7.0 PBS after the antigen–antibody reaction. The detection steps are described in Figure 1b. With the addition of 0.5 increment of CA125 concentration, the peak current increased (Figure 2). The reason might be the fact that the amount of MMBs on the electrode surface increased with CA125 concentration according to the sandwich-type immunoassay format. The electron mediation of Fc-D acted as the bridge to provide an electrical contact or a pathway of electron transfer between the immobilized biomolecules and the base electrode.

To accomplish ultrahigh sensitivity, a high signal-to-noise ratio is required. A cyclic voltammetric measurement with a sandwich-type immunoassay format was employed to detect CA125 with the synthesized MMBs as labels. The current responses increased with the increment of CA125 concentration in the sample solution after the antigen–antibody interaction in pH 7.0 PBS. The increase of reduction current was proportional to CA125 concentration in the range of 0.5 to 55 U mL^{-1} and the linear regression equation is i_p (μA) = 1.3009 + 1.2031 × $C_{[CA125]}$ (U mL^{-1}) with a detection of 0.05 U mL^{-1} (Figure 2B) at a signal to noise ratio of 3δ (where δ is the standard deviation of a blank solution, $n = 15$) ($R^2 = 0.998$).

The reproducibility of the electrochemical immunosensor was evaluated by intra- and interassay coefficients of variation (CVs). The intra-assay precision of the analytical method was evaluated by analyzing 4 concentration levels 5 times per run. The CVs of intraassay with this method were 5.8, 7.3, 4.1, and 6.2% at 1.0, 10, 20, and 40 U mL^{-1} of CA125, respectively. Similarly, the interassay CVs on five immunosensors were 6.7, 4.9, 7.5, and 5.6% at 1.0, 10, 20, and 40 U mL^{-1} of CA125, respectively. Thus, the precision and reproducibility of the proposed immunosensor was acceptable.

To investigate the specificity of the proposed immunosensor, α-1-fetoprotein (AFP), CA 19-9, CEA, and BSA were used in this study. Amperometric responses of the proposed immunosensor in 1.0, 10, 20, and 40 U mL^{-1} of CA125 solutions containing interfering substances of different concentrations were assayed, and the CVs values were 3.7–9.3%, 3.2–9.5%, 2.8–9.1%, and 3.4–8.7%, respectively. So the selectivity of the as-prepared immunosensor was acceptable. The stability of the immunosensor was examined. When the synthesized MMSs were stored at 4 °C, it retained 90.4% of its initial response after a storage period of 21 days. The slow decrease of response seemed to be related to the gradual deactivation of the immobilized antibody incorporated in the composite.

To test for robustness of the method and occurrence of CA125, 45 clinical serum specimens were assayed by the developed immunoassay method and the commercially available ELISA method, respectively. The regression equation (linear) for these data is as follows: $y = 1.271 + 0.9891x$ ($R^2 = 0.991$) (x axis, by the as-prepared immunoassay; y axis, by ELISA). These data show that there is no significant difference between the results given by the two methods.

In summary, by using the newly redox-active MMBs as labels, the sensitivity of the sandwich-type electrochemical immunoassay could be improved. The main advantages of this study is that the Fc-D molecules with a globular shape and branched structure could increase the immobilized amount of biomolecules, and avoid the addition of electron mediator in the sample solution. The potential of this method for application is simple and efficient.

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