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Enzyme–antibody dual-film modified gold nanoparticle probe for ultrasensitive detection of alpha fetoprotein

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ABSTRACT

In this study, we designed a comprehensive strategy for the ultrasensitive detection of alpha fetoprotein (AFP) with high specificity using gold nanoparticle (AuNP)-based enzyme-linked immunosorbent assay (ELISA). A dual-film modified probe was synthesized by coating AuNPs with horseradish peroxidase (HRP) on its surface. Anti-AFP monoclonal antibody (McAb) was immobilized on the surface of the enzyme using glutaraldehyde cross-linking method. AuNPs, employed as support for the immobilization of HRP. HRP was used not only as the enzymatic-amplified tracer but also as a bridge for loading McAb. The limit of detection was 2 ng mL^{-1}. The developed probes can provide an alternative approach with high sensitivity and a simple process similar to that of the traditional HRP-McAb based ELISA for the ultrasensitive detection of AFP in serum.

1. Introduction

Serum alpha fetoprotein (AFP) has been extensively used as a biomarker for liver cancer, which is the second leading cause of cancer death in male worldwide [1]. Additionally, hepatocellular carcinoma (HCC) surveillance using ultrasound combined with AFP measurement is widely applied in practice. Strategies that enhance the sensitivity of existing surveillance tests can improve the feasibility of the early detection of HCC and may provide a more effective method for HCC surveillance [2]. Therefore, a sensitive detection and an accurate analysis of AFP concentrations are important.

Various analytical techniques, such as electrochemiluminescence immunoassay [3], radioimmunoassay [4], imaging ellipsometry [5], and localized surface plasmon-coupled fluorescence [6] have been developed to determine AFP in human serum. These techniques are sensitive and highly specific for AFP detection. However, these methods are unsuitable for a low-cost, convenient, and rapid determination of AFP because they require skilled analysts and delicate instruments.

ELISA, which is based on antibody–antigen immunoreactions, is a powerful method in biological studies because of its convenient operation, capability of simultaneous measurement of large sample size, and low cost [7]. This approach is also the main method for measuring AFP. However, higher sensitivity is required for a precise diagnosis in clinical applications.

Colloidal gold nanoparticles (AuNPs) have earned increasing attention in recent years in bioanalytical application including clinical diagnostics, point-of-care testing and therapeutic research as well as food safety and environmental monitoring [8]. One major merit of using AuNPs is that one can control and tailor their properties in a very predictable manner to meet the needs of specific application [9,10]. Therefore, AuNPs are extensively used for signal amplification in bioanalytical applications [11]. There are two main trends were well-established, one is utilizing the intrinsic properties of AuNPs such as color [12], localized surface plasmons [13], fluorescence resonance energy transfer [14], and many others. Another is carrying numerous biomolecules (e.g. antibody and enzyme molecule) and therefore generated a significant increase in signal [15]. Gao and co-workers designed a new ELISA strategy for highly sensitive colorimetric detection of prostate-specific antigen (PSA) by using AuNPs as the carriers of palladium nanostructures [16]. Li et al. synthesized a dual labeled probe by coating AuNPs with McAb and HRP on their surface. Based on the probe, an immunoassay was ultrasensitive detection of κ-casein in bovine milk samples [17]. The same amplification technique also has been demonstrated in immunomagnetic beads separation [18].

In this study, AuNPs were employed to synthesize an ultrasensitive probe by dual-film assembly with HRP and anti-AFP

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monoclonal antibody (McAb) on the AuNP surface. The introduction of dual-film modified AuNPs into ELISA resulted in an ultrasensitive signal amplification to quantitatively analyze AFP.

2. Materials and methods

2.1. Chemicals and reagents

Chloroauric acid ($HAuCl_4$) was obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Polystyrene 96-well microtiter plates were obtained from Jet Bio-Filtration Products, Co., Ltd. (China). Mouse anti-AFP McAb was procured from Aoke Biotech Co., Ltd. (China). Rabbit anti-AFP polyclonal antibody (PcAb) was procured from Wuhan Huamei Biotech Co., Ltd. (China). HRP-conjugated mouse anti-AFP antibody was obtained from Sigma-Aldrich (USA). AFP from human fetal cord serum and HRP were purchased from Sino Biological Inc. (China). 3,3′,5,5′-tetramethylbenzidine (TMB), H_2O_2, and Tween-20 were supplied by Shanghai Macklin Biochemical Co., Ltd. (China). Alkaline phosphatase (ALP), osteopontin (OPN) and ovalbumin (OVA) were procured from Yeasen Biological Technology Co., Ltd. (China). The various sera were obtained from CellMax Co., Ltd. (China). All other chemicals were of analytical grade and obtained from Aladdin Co., Ltd. (China).

2.2. Solutions and buffers

Phosphate-buffered saline (PBS, pH 7.4) containing 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO_4, and 2 mmol L^{-1} KH_2PO_4. PBS solution with 0.05% Tween-20 (v/v) (PBST) was used as washing buffer. Coating buffer was 50 mmol L^{-1} carbonate buffer (pH 9.5). TMB solution consisted of 50 mmol L^{-1} sodium citrate buffer (pH 5.0) containing 0.01% (w/v) TMB and 0.005% (v/v) H_2O_2. The stop solution was 2 mol L^{-1} H_2SO_4. Deionized water (18 MΩ cm at 25 °C) was used throughout the experiments.

2.3. Preparations of AuNPs

AuNPs were prepared according to the chemical reduction method by Frens [19] with slight modification. All glassware was thoroughly cleaned in aqua regia [3v HCl/1v HNO_3], rinsed in deionized water, and oven-dried prior to use. Then, 2 mL of 1% $HAuCl_4$ solution in 200 mL of deionized water was boiled thoroughly with continuous stirring, and 4 mL of 1% trisodium citrate solution was quickly added under vigorous stirring. The solution color changed from gray to purple to wine-red. The solution was boiled for another 10 min. The heating source was removed, and the solution was continuously stirred for another 15 min. The solution was cooled to room temperature and stored in a dark bottle at 4 °C.

2.4. Dual-film modified AuNPs

The pH of AuNP solution was adjusted to 8.6 with 0.1 M Na_2CO_3. Then, 200 μL of 1 mg/mL HRP solution was added into 10 mL of the AuNP solution under agitation for 20 min. The solution was allowed to stand at 25 °C without mixing for 2 h. Then, 100 μL of 1% (v/v) glutaraldehyde was added and incubated at 4 °C for 12 h to produce aldehyde groups on the enzymatic surfaces. The mixing solution was centrifuged at 9000 rpm for 20 min to remove unconjugated enzyme molecules and excess glutaraldehyde. The precipitate was resuspended in 10 mL of 10 mmol L^{-1} carbonate buffer (pH 8.6). Anti-AFP McAb (1 mg/mL, 5–30 μL) was added into the suspension solution under gentle stirring. The resultant solution was incubated for 4 h at 25 °C. Then, 100 μL of 0.1 mol L^{-1} glycine was added, and the solution was incubated for another 1 h to seal the side residual aldehydes. The probes were purified by centrifuging at 9000 rpm for 20 min to move unconjugated McAb and excess glycine. Following the removal of the supernatant, the red precipitate was washed once with PBS buffer by successive centrifugation and redispersion and then finally was suspended in 10 mL of PBS buffer (containing 1% BSA). The probes were then incubated at 4 °C for 24 h to seal non-specific sites and increase the stability. The dispersity and diameters of the modified AuNPs were determined by a transmission electron microscopy (TEM, JEM-2100) and ultraviolet-visible (UV-vis) spectroscopy (Cary 60). The hydrodynamic diameters of the modified AuNPs were analyzed by dynamic light scattering (DLS, LS-609).

2.5. Detection using ELISA

The AuNP probe was synthesized using HRP and anti-AFP McAb (Fig. 1A). Compared with traditional McAb–HRP-based ELISA, the color density was amplified by the AuNP probe heavily loaded with HRP molecules (Fig. 1B). The assay was performed as follows: 100 μL of anti-AFP PcAb (10 μg mL^{-1}) was added to microtiter plates. The plates were washed thrice with PBST after overnight incubation at 4 °C. Then, 200 μL of 1% BSA was added to the plates, and the plates were incubated for another 1 h at 37 °C. Another washing step was performed, and 50 μL of the sample solution and 50 μL of the AuNP probe were added to each well. The solutions were incubated at 37 °C for 0.5 h. The plates were again washed to remove any unconjugated probe. Then, 100 μL of TMB solution was added, and the solutions were incubated for 5 min at room temperature. The colorimetric reaction was terminated by adding 50 μL of the stop solution. Absorbance was measured at 450 nm using a model 550 microplate reader (Bio-Rad).

3. Results and discussion

3.1. Characterization of AuNP probe

In order to confirm that the probe was indeed successful synthesized, we test the particles during the synthesis process by TEM. The shape of bare AuNPs was spherical and monodispersed, and the average size was 17 nm in diameter (Fig. 2A). Fig. 2B shows HRP fixed to the AuNP surface, the protein halo around the AuNP indicated the successfully modification of AuNP. The TEM imagine of final probe was displayed in Fig. 2C, the probe was also in a colloid stability and monodispersion. Although the thick of protein halo was no obvious increased, this can be explained by considering that analysis by TEM the proteins chains are partially shrinkage during the sample drying process, and make the thin antibody membrane thinner. To further study the hydrodynamic diameters of bare AuNPs, HRP coated AuNPs and the final probes, DLS measurements were conducted (Fig. 2D). In the process of surface modification, the hydrodynamic diameters of particles was increased from 22 nm to 31 nm and then to 37 nm. The reasonable increase in the hydrodynamic diameters after the step-by-step modification process suggests that the proteins were effectively coupled with the particles. The particle size of the bare AuNPs detected by TEM (ca.17 nm) was smaller than that determined by DLS (22 nm) because of the size analysis by DLS is based on particle ensembles, and the AuNPs aggregate in the solution [20]. The DLS mode intensities yielded hydrodynamic diameters values that were systematically larger than those calculated by TEM in protein coated AuNPs. This phenomenon can be explained by considering that the proteins are partially extended under the conditions in DLS measurements. On the contrary, they are collapse in the dried state during TEM measurement [21,22]. Furthermore, both TEM and DLS results were in accord to show the AuNPs were successfully