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Human alpha-fetal protein immunoassay using fluorescence suppression with fluorescent-bead/antibody conjugate and enzymatic reaction

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ABSTRACT

The aim of the study was to develop a simple and rapid immunoassay using fluorescent microbeads and enzyme–substrate reactions to measure alpha-fetal protein (AFP) concentrations. We demonstrated the functionality of the fluorescent immunosensor using antibody-conjugated fluorescent latex beads (AB-FLBs) and horseradish peroxidase (HRP) to catalyze a reaction, where the products would precipitate and suppress the fluorescence of AB-FLBs. First, the AB-FLBs were incubated with antigen, biotinylated antibodies (bABs), and streptavidin-HRP (SAv-HRP) to form a sandwich-type immunoreaction. The mixture was then filtered through a membrane to concentrate the beads on a small area. After washing to remove unbound bABs and SAv-HRP, a chromogenic HRP substrate and H_2O_2 were added to form precipitates on the FLB surface. The suppression of the fluorescence was measured with a fluorescent image analyzer system. Under optimized conditions, AFP could be measured at concentrations as low as 1 pg mL^{-1} with a dynamic range up to 100 ng mL^{-1}.

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1. Introduction

Immunoassays, those based on the highly specific molecular recognition of antigen–antibody binding reaction, have become indispensable for disease diagnosis and clinical research. There have been many different kinds of immunoassay, including radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, chemiluminescence immunoassay, and bioluminescence immunoassay (Diamandis and Christopoulos, 1996). Although immunoassays are highly sensitive, many conventional immunoassays, such as immunoradiometric assays, single radial immunodiffusions, and enzyme-linked immunosorbent assays (ELISA), have some limitations, including the short shelf life of radiolabeled antibodies, radiation hazards, complicated washing steps, a long analysis time, and the need for skilled operators (Ju et al., 1999; Palmer and Miller, 1995; Nilsson et al., 1992). Therefore, new techniques, such as electrochemistry (Chen et al., 2002), chemiluminescence (Konry et al., 2005), piezoelectricity (Zuo et al., 2004), Raman scattering (Ma et al., 2014), and surface plasmon resonance (Kurita et al., 2006; Wu et al., 2013), have been developed and have attracted widespread interest due to their simplicity and specificity. However, these methods are limited by the need for a solid phase for antibody immobilization and have complicated blocking, washing, and antigen–antibody reaction steps.

ELISA, based on antigen–antibody binding and the enzymatic activity reporter, has been widely used for a biological and medical analysis from 1971 (Engvall and Perlmann, 1971; VanWeeman, 1971). The fluorescence immunoassay can improve the sensitivity to 10–1000 times higher than the ELISA (Diamandis and Christopoulos, 1996). Quantum dots (Q-dots) have widely applied as the label for immunoassays (Peng et al., 2009; Kuang et al., 2011, 2013), but their high costs are a deterrent to widespread use. In this study, we combined an absorbance immunoassay with a fluorescence immunoassay for the simple and fast detection and diagnosis of various medical conditions and diseases.

Alpha-fetal protein (AFP) is widely used as a marker of germ cell tumors, hepatocellular carcinoma, and liver carcinoma (Alpert et al., 1971; Okuda, 1986; Wang and Xie, 1999). AFP is normally synthesized by the liver, yolk sac, and gastrointestinal tract of the human fetus, but it is also often found at high levels in the sera of adults with certain malignancies. In addition to certain tumors,

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elevated serum AFP levels may be present in ataxia-telangietasia syndrome, herediatary tyrosinemia, cirrhosis, alcoholic hepatitis, and viral hepatitis. Although AFP is not a specific genetic marker for malignancies, it may be used to monitor the effectiveness of surgical and chemotherapeutic treatments of hepatomas and germ cell neoplasms (Mosby, 2009). Numerous immunoassays for detecting AFP have been recently developed. Huang et al. (2011) developed a one-step AFP immunoassay using imaging ellipsometry. Jiang et al. (2010) researched an electrochemical immunosensor with multi-walled carbon nanotubes using the amperometric method. Giannetto et al. (2011) developed a voltametric immunosensor using nanocomposite materials that contained an antibody/antigen/antibody-horseradish peroxidase (HRP) sandwich on a glass carbon electrode surface. The conventional AFP immunoassay based on a sandwich ELISA has disadvantages, such as the requirement for a solid phase to immobilize the bioreceptor, and multiple time-consuming and complicated blocking and washing steps. Here, we developed an alpha-fetal protein (AFP) immunoassay based on the sandwich ELISA method with fluorescent latex beads (FLBs) and enzyme-catalyzed precipitation to suppress fluorescence. For our assay, we used antibody-conjugated FLBs (AB-FLBs) and filtered AB-FLB/AFP/biotinylated AB/streptavidin-HRP complexes (AB-FLB/AFP/bAB/SAv-HRP) on a membrane. The antigen–antibody reaction was detected by suppressing sandwich immunoassayed fluorescent latex beads using the precipitation of the enzyme-substrate reaction.

2. Materials and methods

2.1. Materials

Carboxylate-modified polystyrene yellow–green FLBs (2.0 μm; λex: ~470 nm, λem: ~505 nm), horseradish-peroxidase (HRP), alkaline-phosphatase (ALP), 4-chloro-1-naphthol (CN), 3,3′-diaminobezidine (DAB), ntirotetrazolium blue chloride (NBT), 5-bromo-4-chloro-4-indolyl phosphate disodium salt (BCIP), polyvinylpyrrolidone (PVP), N-hydroxysuccinimide (NHS), streptavidin-alkaline phosphatase (SAv-ALP), human serum (HS), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Thermo Sciences (Rockford, IL, USA). Horseradish peroxidase conjugated streptavidin (SAv-HRP) was purchased from Calbiochem (CA, USA). Alpha-fetal protein (AFP), anti-AFP, and biotinylated anti-AFP were purchased from Meridian Life Science (ME, USA). A 10 G surfactant was obtained from Fitzgerald Industries (MA, USA). 0.45 μm pore sized membranes and syringe filters of PVDF were purchased from EMD Millipore (MA, USA) and Whatman™ GE Healthcare (England, UK). Human alpha fetoprotein ELISA kit was purchased from Alpha Diagnostic International (TX, USA).

2.2. Extinction of enzyme–substrate reaction products

For the CN precipitation, 1 μL of 1 mg mL^{-1} SAv-HRP was reacted with 1 mM CN and 1 mM H_2O_2 in a 1 mL PBS buffer for 30 min. For the DAB precipitation, 1 μL of 1 mg mL^{-1} SAv-HRP was reacted with 1 mM DAB and 1 mM H_2O_2 in a 1 mL PBS buffer for 30 min. For the NBT/BCIP precipitation, 1 μL of 1 mg mL^{-1} SAv-ALP was reacted with 1 mM BCIP and 1 mg mL^{-1} NBT in 1 mL of a 0.1 M tris–HNO_3 pH 9.8 buffer for 30 min. The extinction of the reacted mixture was measured with a DU 800 UV/Vis spectrophotometer (Beckman Coulter, USA). To comparison with the fluorescent latex beads, the excitation and emission wavelength of FLBs were measured with a LS55 fluorescence spectrometer (Perkin Elmer, USA).

2.3. Preparation of BSA-conjugated fluorescent latex beads (BSA-FLBs)

Carboxylate fluorescent latex beads were diluted 10^3-fold, and reacted with 0.1 mL of 0.4 M EDC and 0.1 mL of 0.1 M NHS for 30 min during rotation at 10 rpm. Then 0.1 mL of 10% BSA in 1 × PBS was added to the activated fluorescent latex beads. The mixed solution was spun down at 3000 rpm for 10 min using centrifuge. After that, the supernatant was removed, and 1% BSA in PBS was added to the settled beads for washing. The washing stage was carried out twice.

2.4. Substrate adsorption on bare membrane and fluorescent latex beads

The 0.45 μm pore-sized PVDF membrane is printed using ColorQube 8570 solid ink printer (Xerox, Japan). The pattern has the square size of 10 mm × 10 mm, and the empty circle of 1 mm ϕ in the center of a square pattern. The membrane was blocked with a 10% 10 G surfactant and 20% PVP in distilled water. The prepared BSA-conjugated fluorescent latex beads (BSA-FLB) were diluted in 1 × PBS to 10^{-4} × BSA-FLB and filtered on the printed membrane. After BSA-FLB filtration using a Büchner funnel and aspirator, the sample was washed with distilled water and dried at 4 °C. The BSA-FLB filtered membrane was reacted with 1 mM CN and 1 mM H_2O_2 in a 10 mM phosphate buffer pH 7.4, 1 mM DAB and 1 mM H_2O_2 in a 10 mM phosphate buffer pH 7.4, or a 1 mM BCIP and 1 mg mL^{-1} NBT in 0.1 M tris–HNO3 pH 9.8 buffer for 10 min. The reacted membranes were washed with distilled water. The substrate adsorption on BSA-FLB and the membrane was measured with an LAS 3000 luminescence image analyzer (Fuji, Japan).

2.5. Preparation of enzyme-conjugated and fluorescent latex beads

0.001 × carboxylate fluorescent latex beads were reacted with 0.1 mL of 0.4 M EDC and 0.1 mL of 0.1 M NHS for 30 min during rotation at 10 rpm. Then 50 μL of 1 mg mL^{-1} peroxidase was added to the activated fluorescent latex beads solution and reacted for 1 h. 0.1 mL of 10% BSA in 1 × PBS was added to the peroxidase-conjugated fluorescent latex beads to block unreacted carboxylate-functional groups of the surfaces of the fluorescent latex beads. The mixed solution was spun down at 3000 rpm for 10 min using centrifuge. After that, the supernatant was removed, and 1% BSA in PBS was added to the settled beads for washing. The washing stage was carried out twice. ALP-conjugated fluorescent latex beads were prepared with the same process as the HRP-conjugated fluorescent latex beads.

2.6. Enzyme–substrate reaction on membrane using enzyme-conjugated fluorescent latex beads

The prepared peroxidase-conjugated fluorescent latex beads (HRP-FLB) were diluted in 1 × PBS to 10^{-4} × HRP-FLB and filtered on a printed membrane. After HRP-FLB filtration using a Büchner funnel and aspirator, the sample was washed with distilled water and dried at 4 °C. The HRP-FLB filtered membrane was reacted with 1 mM CN and 1 mM H_2O_2 in a 10 mM phosphate buffer pH 7.4, or 1 mM DAB and 1 mM H_2O_2 in 10 mM PB pH 7.4 for 10 min. The prepared alkaline phosphatase-conjugated fluorescent latex beads (ALP-FLB) were filtrated with the same process of HRP-FLB. The filtered ALP-FLB was reacted with 1 mM BCIP and 1 mg mL^{-1} NBT in 0.1 M tris-HNO3 pH 9.8 buffer for 10 min on the printed membrane. The fluorescent suppression of HRP-FLB was measured with an LAS 3000 luminescence image analyzer.

2.7. Preparation of antibody-conjugated fluorescent latex beads (AB-

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