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Competitive-type displacement reaction for direct potentiometric detection of low-abundance protein

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ABSTRACT

Prostate-specific antigen (PSA), one of the indications of possible prostate malignancy, is used as a biomarker for the diagnosis and prognosis of prostate cancer. Herein, we develop a new homogeneous potentiometric immunoassay for sensitive detection of low-concentration PSA without the need of sample separation and washing step. Two nanostructures including positively charged polyethyleneimine-poly(styrene-*co*-acrylic acid) (PEI-PSAA) nanospheres and negatively charged gold nanoparticles conjugated with anti-PSA antibody (Ab-AuNP) were first synthesized by using mulsifier-free emulsion copolymerization and wet chemistry method, respectively. Thereafter, the as-prepared PEI-PSAA was used as a pseudo hapten for the construction of immunosensing probe based on an electrostatic interaction between PEI-PSAA and Ab-AuNP. Upon target introduction, the added PSA competed with PEI-PASS for Ab-AuNP based on a specific antigen–antibody interaction, and displaced Ab-AuNP from PEI-PASS. The dissociated PEI-PASS was captured through the negatively charged Nafion- modified electrode, thereby resulting in the change of membrane potential. The fabrication process was characterized by using high-resolution transmission electron microscope (HRTEM), scanning electron microscope with energy-dispersive X-ray spectroscopy (SEM-EDX), surface plasmon resonance (SPR) and dynamic laser scattering (DLS) technique. Under optimal conditions, the output signal was indirectly proportional to the concentration of target PSA in the sample and exhibited a dynamic range from 0.1 to 50 ng/mL with a detection limit (LOD) of 0.04 ng/mL. Intra- and inter-assay coefficients of variation (CVs) were 6.8 and 7.5%, respectively. In addition, the methodology was evaluated for analysis of 12 clinical serum samples and showed good accordance between the results obtained by the developed immunosensing protocol and a commercialized enzyme-linked immunosorbent assay (ELISA) method.

1. Introduction

Immunoassay and immunosensor are usually utilized as a powerful tool for quantitative monitoring of clinically important compounds in the complex biological hierarchy (Lin et al., 2013; Jeong et al., 2013) and substances in the environment (Date et al., 2013; Zhang et al., 2012a; Tang et al., 2013) because of highly sensitive and specific antigen–antibody reaction. Various methods and strategies have been reported for this purpose based on different signal-transducer principles, e.g. fluorescence (Chen et al., 2012), electrochemistry (Wang et al., 2013a, 2013b), electrochemiluminescence (Liang et al., 2012), and colorimetric assay (Wang et al., 2013a, 2013b; Zhou et al., 2012). Recently, ongoing efforts have been made worldwide to develop and improve the clinical immunoassay with the aim of manufacturing portable and affordable diagnostic devices (Zhang et al., 2013). An alternative approach that is based on an electrochemical principle and does not require sample separation and washing step would be advantageous because of simple instrumentation and easy signal quantification (Tang et al., 2007).

Homogenous immunoassay commonly involves in the immobilization of capture antibody on the nano/microbeads and takes place in the solution, thus allowing the integration of multiple liquid handling processes (Limoges and Degrand, 1993; Kim et al., 2005; Sakashita et al., 1995). Most recently, Akhavan-Tafti et al. (2013) reported a new homogeneous immunoassay method featuring the use of specific binding members separately labeled with an acridan-based chemiluminescent compound and a peroxidase. Without any separation steps, the signal readout could be generated upon addition of a trigger solution. Hu et al. (2012) also designed a separation-free, electrochemical assay format with direct readout that was amenable to highly sensitive and selective quantitation of a wide variety of target proteins.

For the successful development of a good homogeneous immunoassay, the construction of immunosensing platform is very crucial. Intrinsically conducting polymers have shown great

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potential for a variety of applications including sensor, anticorrosion coating, battery, capacitor, actuator, and optical device (Bubnova et al., 2012; Talemi et al., 2013). Polystyrene shows promise for commercial applications because of its simple synthesis, high conductivity, environmental stability, and biocompatibility (Uyar et al., 2010; Nakamura et al., 2005). Polystyrene composites have been shown to exhibit easy modification which could lead to biomedical applications such as cell adhesion studies (Palacios et al., 2013) or thermal ablative cancer therapy (Shi et al., 2005). Recent research has looked to develop innovative and powerful novel functionalized nanometer-sized polystyrene microspheres, controlling and tailoring their properties in a very predictable manner to meet the needs of specific applications. Lin et al. (2012) exploited polystyrene microspheres as the labels to build ultrasensitive electrochemical immunosensor for the detection of proteins. Nafion, as a perfluorosulfonate ion-exchange polymer, has been extensively used for preparation of chemically modified electrodes and the construction of ion-exchange membranes (Ensing et al., 2013; Dong et al., 2010). The unique properties of using Nafion are (i) the outstanding chemical and thermal stabilities, (ii) preconcentrating the electroactive and photoactive cations even from dilute solution, and (iii) the multiphase structure consisting of a fluorocarbon hydrophobic phase, a hydrophilic ionic cluster, and interfacial regions (Nieh et al., 2013; Hseih et al., 2014; Siracusano et al., 2013; Matos et al., 2013). The cationic molecules are exchanged into the Nafion film by both electrostatic and hydrophobic interactions due to the sulfonate head groups ($-SO_3^-$) and a fluorocarbon chain (Ladewig et al., 2007; Chen et al., 2008).

Herein, we report the proof-of-concept of a new, simple, low-cost, and powerful homogeneous potentiometric immunoassay for sensitive detection of low-abundance proteins based on target-induced competitive-type displacement reaction between polyethyleneimine- functionalized poly (styrene-*co*-acrylic acid) microsphere (PEI-PSAA) and nanogold particle- labeled capture antibody (Ab-AuNP). Prostate-specific antigen (PSA), also known as gamma- seminoprotein or kallikrein-3 (KLK3), is a glycoprotein enzyme encoded in humans by the *KLK3* gene, which is used as a model analyte for the development of the homogeneous immunoassay. First, the as-synthesized Ab-AuNP with negative charge is attached onto the surface of positively charged PEI-PSAA by the opposite-charged adsorption technique, which is used as an immunosensing probe for the detection of target PSA. Upon addition of target analyte, target PSA competes with PEI-PSAA for Ab-AuNP on the basis of the specific antigen–antibody reaction, and displaces the Ab-AuNP from the PEI-PSAA. The dissociated PEI-PSAA can be captured by the negatively charged Nafion-modified electrode, thus resulting in the change of membrane potential. By monitoring the shift in the potential by using a potentiometry, we can indirectly determine the concentration of target PSA in the sample.

2. Experimental

2.1. Materials and reagents

Mouse anti-human monoclonal prostate-specific antibody (anti-PSA, designated as Ab, 0.1 mg/mL) was purchased from Amyjet Scientific Inc (Abcam product, Wuhan, China). PSA standards with various concentrations were obtained from Biocell Biotechnol. Co., Ltd. (Zhengzhou, China). Polyethyleneimine (PEI, branched, MW 10,000, 99 wt%), polyethylene glycol (PEG, MW 6000), Nafion (5 wt%), styrene, acrylic acid, and $HAuCl_4 \cdot 4\,H_2O$ were achieved from Alfa Aesar®. All other reagents were of analytical grade and were used without further purification. Ultrapure water obtained from a Millipore water purification system ($\geq$ 18 MΩ, Milli-*Q*) was used in all runs. Phosphate-buffered saline (PBS, 0.1 M, pH 7.4) solution was prepared by mixing 0.1 M $NaH_2PO_4$and 0.1 M Na_2HPO_4, and 0.1 M KCl was added as the supporting electrolyte. Clinical serum samples were made available by Fujian Provincial Hospital, China.

2.2. Preparation of polyethyleneimine-functionalized poly(styrene-co-acrylic acid) microspheres (PEI-PSAA)

Before modification, poly(styrene-*co*-acrylic acid) microspheres (PSAA) were synthesized according to the literatures with a little modification (Lin et al., 2012; Zhang et al., 2012b). Briefly, 150 mL of ultrapure water was initially injected into a cleaned three-necked flask submerged in an oil bath, and then the solution was purged with nitrogen for 1 h. Following that, styrene (5.0 g) and acrylic acid (1.0 g) were simultaneously added into the three-necked flask with stirring at 70 °C under the protection of nitrogen. Upon addition of $K_2S_2O_8$ (0.1 g, 5 mL) solution into the mixture, the emulsifier-free emulsion copolymerization of the styrene and acrylic acid was taken place. After refluxing for 8 h, the resulting PSAA suspension was centrifuged for three times at 8000 rpm for 20 min and washed with distilled water. Afterward, PSAA microspheres were functionalized with the polyethyleneimine (PEI) by simply mixing together. Finally, the as-prepared PEI-PSAA microspheres were dispersed into distilled water with a fixed concentration of 20 mg/mL, and stored at 4 °C for further use.

2.3. Synthesis of nanogold-labeled anti-PSA antibody (Ab-AuNP)

First, gold nanoparticles (AuNP) with 16 nm in diameter were synthesized by reduction of chlorauric acid with trisodium citrate according to our previous reports (Zhang et al., 2012c; Gao et al., 2013). Initially, 1 mL of 1 wt% $HAuCl_4$ aqueous solution was added to 99 mL of distilled water, and then the mixture was heated to 100 °C. Following that, 2.5 mL of sodium citrate (1 wt%) was quickly dropped into the boiling solution. During this process, the $Au^{(III)}$ was reduced to zero-valent Au^0 (Ojea-Jiménez, Puntes, 2009; Ambrosi et al., 2007). The as-synthesized gold colloids were characterized by using transmission electron microscopy (TEM), and the mean size was ~16 nm (Tang et al., 2011).

Next, the as-prepared gold colloids were used for the labeling of anti-PSA antibody similar to our previous report (Gao et al., 2013). Initially, 1 mL of 16-nm gold colloids ($C_{[Au]} \approx 24$ nM) was adjusted to pH 8–9 by using Na_2CO_3, and then 100 μL of 0.1 mg/mL anti-PSA antibody was added to the colloids. The mixture was gently shaken several times, and stored at 4 °C for incubation overnight. Following that, the mixture was centrifuged (14 000 rpm) for 30 min at 4 °C to remove the excess antibody. The obtained soft sediment (i.e. nanogold-labeled anti-PSA antibody, designated as Ab-AuNP) was resuspended into 1 mL of PBS (pH 7.4) containing 1 wt% BSA and stored at 4 °C for further usage.

2.4. Electrochemical measurement

A gold electrode (2 mm in diameter) was polished repeatedly with 0.3 and 0.05 μm alumina slurry, followed by successive sonication in distilled water and ethanol for 5 min and dried in air. Before modification, the gold electrode was initially cleaned with hot piranha solution (a 3:1 mixture of H_2SO_4 and H_2O_2. *Caution*!) for 10 min, and then continuously scanned within a potential range from −0.3 to 1.5 V in freshly prepared deoxygenated 0.5 M H_2SO_4 until a voltammogram characteristic of the clean gold electrode was established. Following that, 5 μL of 1 wt % Nafion ethanol solution was thrown on the gold electrode and then removed and parched under an infrared light for 20 min.