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A doubly amplified electrochemical immunoassay for carcinoembryonic antigen

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ABSTRACT

An ultrasensitive electrochemical immunoassay (EIA) for the detection of carcinoembryonic antigen (CEA) is described in this report. The assay involves utilizing enzyme-catalyzed deposition of a redox polymer and electrocatalytic oxidation of ascorbic acid (AA) by the deposited redox polymer, a dual-amplification scheme to enhance analytical signals. Briefly, CEA capturing antibody and redox polymer anchoring agent were covalently immobilized on a gold electrode. After incubating with CEA, the electrode was treated in detection antibody–glucose oxidase conjugate solution. Thereafter, it was dipped into the redox polymer solution. Upon the addition of glucose, the redox polymer was enzymatically reduced and deposited on the electrode surface. The deposited redox polymer exhibits excellent electrocatalytic activity towards the oxidation of AA. Consequently, CEA could be quantified amperometrically. This electrochemical immunoassay combines the specificity of the immunological reaction with the sensitivity of the doubly amplified electrochemical detection.

1. Introduction

It is clinically desirable to develop portable protein detection devices which would enable clinicians to measure a few key proteins at the point of care such as small clinical laboratories and doctors' offices (Macbeath, 2002; Mitchell, 2002; Emili and Cagney, 2000). Unlike DNA assays, where high specificities can easily be predetermined by simple chemical synthesis of oligonucleotide capture probes and high sensitivity can be realized by employing a polymerase chain reaction (PCR) (Sassolas et al., 2008), the lack of proteome-wide bioamplification strategy has been one of the bottlenecks to ultrasensitive protein assays (Macbeath, 2002; Mitchell, 2002; Emili and Cagney, 2000). Among a wide variety of protein assays investigated, sandwich immunoassay with optical detection is the most dominant since it does not require the analyzed sample to be labeled. This greatly simplifies sample preparation and shortens assay time. The popularity of the sandwich immunoassay advanced greatly in the 1970s with the introduction of enzyme-linked immunosorbant assay (ELISA) by Engvall and Perlman (1974). It has become a standard technology in clinical laboratory.

Although optical detections have primarily been employed in ELISA, electrochemical detections are more advantageous for such assays owing to their high sensitivity and high portability (Rossier and Girault, 2001). Furthermore, for opaque or optically dense matrices electrochemical detections are superior. For example, electrochemical measurements can be made on whole blood without interference from blood cells, other proteins, and fat globules (Hirsch et al., 2003; Yao et al., 1995). Electrochemical immunoassays (EIA) have therefore been studied as an attractive alternative to optical immunoassays (Bakker and Telting-Diaz, 2002; Diaz-Gonzalez et al., 2005). Of the many proposed EIA, those employing amperometric detections have several additional advantages over other EIA, such as straightforwardness and high sensitivity. Traditionally, amperometric detection is carried out in solution phase, relying on enzyme-mediated solution phase reaction for the formation of electroactive species (Rossier and Girault, 2001; Diaz-Gonzalez et al., 2005; Bakker and Telting-Diaz, 2002). Due to dilution effect, detection limits were usually at ng/mL levels. To lower the detection limits, various amplification strategies, such as substrate-recycling (Bauer et al., 1996) the use of nanoparticulate tags (Wang, 2007; Cui et al., 2007) and magnetic beads (Thomas et al., 2004; Choi et al., 2002), the adoption of DNA amplification techniques, including rolling circle amplification (Zhou et al., 2007) and biobarcode-based PCR (Stoeva et al., 2006), have been proposed to improve the performance of EIA. Nonetheless, new schemes based on coupling the biocatalytic amplification of enzyme tags with additional amplification units and processes are highly desirable for meeting the high sensitivity demands of electrochemical detection of proteins.

In this work we showed an adaptation of a dual-amplification scheme in EIA to further enhance the sensitivity and lower the detection limit. The applicability of the system in protein detection

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was demonstrated with an important colon cancer biomarker, carcinoembryonic antigen (CEA). Different from other EIA previously published, a second amplifier, the redox polymer is deposited on the electrode surface by the enzyme tag (first amplifier, GOx in this case). And subsequently utilizing the deposited redox polymer as an electrocatalyst for the oxidation of ascorbic acid (AA), CEA can be conveniently detected amperometrically. The sensitivity and detection limit of the assay are markedly improved which are superior to solution-phase EIA.

2. Experimental

2.1. Materials and reagents

Unless otherwise stated, chemicals were obtained from Sigma–Aldrich (St Louis, MO) and used without further purification. The redox polymer used in this study was poly(4-vinylimidazole-co-acrylamide) partially imidazole-complexed with $[Os(dmpy)_2Cl]^{2+}$ (PVIA-Os). Synthesis of the redox polymer was described elsewhere (Campbell et al., 2002). The oxidized form of PVIA-Os was used in the protein detection. To demonstrate the "proof-of-principle", CEA was selected as the model protein since rabbit polyclonal antibody and monoclonal antibodies to this cancer biomarker are commercially available. CEA (CD66e, molecular weight ~180 KDa) and its monoclonal antibody (mAb, clone C6G9) were purchased from Sigma. The biotinylation of mAb was done as described in a previous report (Hnatowich et al., 1987). Briefly, the mAb solution was dialyzed overnight at 4 °C against pH 8.3 0.10 M sodium bicarbonate buffer. A 200 μL aliquot of biotin-N-hydroxysuccinimide ester (2.0 mg/mL) dissolved in dimethyl sulfoxide was added to a 1.0 mL aliquot of 1.0 mg/mL mAb. After a 4-h incubation at room temperature on a rotator, the mixture was centrifuged at 5000 rpm for 10 min to remove any precipitates and then extensively dialyzed against phosphate-buffered saline (PBS, pH 7.0) for 24 h at 4 °C with five changes of PBS in between. The dialyzed biotinylated mAb was then stored at −80 °C until use. Avidin–glucose oxidase conjugate (A–GOx) was obtained form victor laboratories (Burlingame, CA 94010).

2.2. Electrode fabrication

To fabricate the working electrode, a titanium adhesion layer of 25–50 Å was first electron-beam evaporated onto a glass slide followed by 750–1000 Å of gold. It was then annealed at 250 °C in air for 3 h. Before mAb immobilization, the gold coated slide was thoroughly cleaned in a freshly-prepared piranha solution (98% H_2SO_4/30% H_2O_2 = 3/1. **Caution**: Piranha solution is a powerful oxidizing agent and reacts violently with organic compounds.) and copiously rinsed with Milli-Q water followed by 10 min sonication in absolute ethanol. The gold surface was then modified immediately after the cleaning step. Initial thiol adsorption was accomplished by immersing the gold substrate in an ethanolic solution of 2.0 mM 11-mercaptoundecanoic acid (MUA) at room temperature. The slide was rinsed with Milli-Q water and activated with 100 mM of 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide (EDC) and 40 mM of N-hydroxysulfosuccinimide (NHS) in water. A patterned 1-mm thick adhesive spacing/insulating layer was assembled on the top of the slide, to form an array of electrodes. The diameter of the individual electrodes was 2.0 mm. Aliquots of 0.10 mg/mL mAb in PBS were applied to the electrode and incubated for 3 h at room temperature. After rinsing with washing buffer (PBS + 0.050% Tween-20), the unoccupied MUA sites were treated in 2.0 mM 1(3-aminopropyl)-imidazole (API) in PBS overnight at room temperature. Bovine serum albumin (BSA) coated electrodes were used as control. The mAb coated electrodes were stable for at least 6 months at 4 °C.

2.3. Characterization

Electrochemical experiments were carried out using a Model 660A electrochemical workstation coupled with a low current module (CH Instruments, Austin, TX). The three-electrode system consisted of the 2-mm-diameter gold working electrode, a miniature Ag/AgCl reference electrode, and a platinum wire counter electrode. PBS was used as the supporting electrolyte.

Immunoreaction and redox polymer deposition were monitored by a quartz crystal microbalance (QCM) cell (CH Instruments Inc., Austin, TX) in a clean environment. The frequency responses were stable within 2.0 Hz in air over a period of 30 min. Quartz crystal resonators employed in QCM experiments were 13.6-mm-diameter 10-MHz AT-cut type with 5.11-mm-diameter gold electrodes on both sides, purchased from Fortiming Corp. (Ashland, MA). Stable frequency responses were taken after washing off unreacted materials and drying in a stream of air. To minimize variation from electrode to electrode, the same electrode was always used for both immunoreaction and redox polymer deposition. The Sauerbrey equation is used for calculating mass change at the quartz crystal resonator (Sauerbrey, 1959):

$$\Delta f = 2f_0^2(\mu_q\rho_q)^{-1/2}\frac{\Delta m}{A} \quad (1)$$

where Δf is the measured frequency shift in hertz due to mass change, f_0 the resonant frequency of the fundamental mode of the resonator, μ_q the shear modulus of quartz (2.95×10^{11} dyne/cm^2), ρ_q the density of the resonator (2.65 g/cm^3), Δm the mass change of the resonator and A electrode surface area. For the 10 MHz resonator used in this study, Eq. (1) predicts that a frequency change of 1.0 Hz corresponds to a mass change of 0.90 ng on the resonator.

2.4. Protein detection

CEA incubation and its electrochemical detection were carried out in five steps, as depicted in Fig. 1. The electrode was placed in a moisture-saturated environmental chamber. Aliquots of CEA solution (2.0 μL) in PBS (pH 7.4) were placed on the electrode and incubated for 30 min. After washing for 10 min in a vigorously stirred PBS and drying, biotinylated mAb/A–GOx (1/2, 5.0 μL) was dispensed onto the electrode and incubated for 10 min. The electrode was washed, dried and a 10 μL aliquot of glucose–redox polymer mixture in PBS (pH 6.0–7.0) was applied onto the electrode and incubated for 30 min. The catalytic response was evaluated by amperometry at a constant potential of 0.15 V in PBS (pH 7.4) containing 5.0 mM AA. In the case of low CEA concentrations, smoothing was applied after each measurement to remove random noises. All incubations and measurements were performed at room temperature. All potentials reported in this work were referred to an Ag/AgCl reference electrode.

3. Results and discussion

3.1. GOx-catalyzed deposition of redox polymer

QCM was first used to investigate the immunoreaction and the redox polymer deposition characteristics (Table 1). Various osmium–bipyridine-based redox polymers were tested for their abilities to form stable deposits during GOx-catalyzed reduction