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Enzyme-free electrochemical immunosensor based on methylene blue and the electro-oxidation of hydrazine on Pt nanoparticles

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ABSTRACT

Enzyme-free electrochemical sensors enable rapid, high sensitivity measurements without the limitations associated with enzyme reporters. However, the performance of non-enzymatic electrochemical sensors tends to suffer from slow electrode kinetics and poor signal stability. We report a new enzyme-free electrochemical immunosensor based on a unique competitive detection scheme using methylene blue (MB), hydrazine and platinum nanoparticles (Pt NPs). This scheme is coupled with a robust immunosandwich format employing a MB-labelled detection antibody as a non-enzymatic reporter. In the presence of the target antigen, surface-immobilized MB consumes interfacial hydrazine thereby diminishing the electro-oxidation of hydrazine on Pt NPs. Thus, the concentration of the antigen is directly proportional to the reduction in the electrochemical signal. For proof-of-concept, this sensor was used to detect *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2), an important malaria biomarker, in unadulterated human saliva samples. Chronocoulometric measurements showed that this platform exhibits pM-range sensitivity, high specificity and good reproducibility, making it well suited for many biosensing applications including noninvasive diagnostic testing.

1. Introduction

Recently, there has been growing interest in the development of enzyme-free electrochemical sensors due to their potential for enhanced stability and reproducibility compared with enzymatic electrochemical sensors. Much effort in this area has focused on the detection of hydrogen peroxide (H_2O_2), glucose and uric acid due to the clinical relevance of these analytes (Chen et al., 2014; Chen et al., 2013a, 2013b). Research has also been carried out to develop enzyme-free electrochemical immunosensors for the detection of protein biomarkers (Tang et al., 2015). While current enzyme-free sensors can offer high sensitivity measurements, many are based on electrochemical reactions that tend to suffer from sluggish electrode kinetics or poor signal stability (Wang et al., 2013a; Si et al., 2013).

The electro-oxidation of hydrazine is a well-studied reaction that has been utilized for various applications including fuel cells and biosensors (Sanabria-Chinchilla et al., 2011; Finkelstein et al., 2016; Rahmana et al., 2005). The electroactivity of hydrazine oxidation can be enhanced through the addition of electrocatalysts such as gold, silver or platinum (Dudin et al., 2011; Ojani et al., 2015; Koçak et al., 2016). Of these metals, platinum (Pt) offers excellent stability and fast reaction kinetics (Tiwari et al., 2013). The oxidation of hydrazine on Pt is a faradaic reaction which generates an electrochemical current:

$$N_2H_4 + Pt \rightarrow N_2 + H_20 \quad (1)$$

In the presence of oxygen, a non-faradaic (no current generation) side reaction takes place between hydrazine and aerial oxygen on the Pt surface resulting in the decomposition of hydrazine into N_2 and H_2O (Chen et al., 2015):

$$N_2H_4 + O_2 \rightarrow N_2 + 2H_2O \quad (2)$$

As the interfacial hydrazine is consumed, the oxidation current decreases accordingly. Aldous et al. further reported that platinum oxide (PtO_2) plays an important role in hydrazine electro-oxidation where the reaction of hydrazine with PtO_2 occurs layer-by-layer and results in a time-dependent deactivation of Pt (Aldous et al., 2011).

The fast electro-oxidation of hydrazine on Pt makes it an excellent candidate for electrochemical biosensors requiring a rapid response. Several groups have demonstrated electrochemical DNA sensors using hydrazine as a catalyst and Pt NPs as a label (Shiddiky et al., 2007; Kwon et al., 2012). While capable of rapid measurements, these sensors require complicated detection protocols or lack the sensitivity needed for detection in raw biological samples. Others have developed

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enzyme-free DNA sensors using methylene blue (MB) as a redox indicator and hydrazine as a substrate (Zhua et al., 2006; Yu and Lai, 2013; Alligrant et al., 2015). However, these methods exhibit lower signal-to-background ratios and longer detection times compared with biosensors utilizing only hydrazine and Pt. While the reduction of MB using various transition metals has been reported (Jana et al., 2000; Ganapuram et al., 2015), the effects of MB reduction on the electro-oxidation of hydrazine in the presence of Pt has yet to be studied or utilized for biosensing purposes.

In this work, a new enzyme-free immunosensor was developed based on a unique, competitive electrochemical scheme between MB, hydrazine and Pt nanoparticles (NPs). We are the first to apply this unique scheme for high sensitivity analytical measurements of protein biomarkers. This biosensor employs a robust immunosandwich format where the capture antibody is immobilized on an ITO electrode and the detection antibody is labelled with MB. In the absence of the target antigen, hydrazine is rapidly electro-oxidized on Pt NPs generating a high electrochemical signal. In the event of antigen-antibody binding, interfacial hydrazine is consumed by surface-immobilized MB in a non-faradaic side reaction which decreases the electrochemical current. This detection scheme offers several advantages including rapid electrokinetics, high sensitivity, and good reproducibility. Furthermore, this sensor offers excellent detection performance even in raw biofluid samples with minimal interference effects.

2. Experimental

2.1. Biochemicals and reagents

(3-Aminopropyl) triethoxysilane (APTES), glutaraldehyde, ethanolamine, bovine serum albumin (BSA), hydrazine monohydrate, chloroplatinic acid, ammonium hydroxide (NH_4OH), hydrogen peroxide (H_2O_2), sodium citrate tribasic dihydrate, ascorbic acid, phosphate-buffered saline (PBS, pH 7.4) and all reagents for buffer solutions were obtained from Sigma-Aldrich (St. Louis, MO). All buffer solutions were prepared using (deionized) DI water (18.3 MΩ-cm^{-1}) generated using a Thermo Fischer Scientific Smart2Pure water purification system. A PBS-BSA solution was prepared by dissolving BSA 1% (w/v) in PBS. Mouse monoclonal anti-*Pf*HRP2 IgM (capture antibody) and mouse monoclonal anti-*Pf*HRP2 IgG (detection antibody) were purchased from ICL, Inc. (Portland, OR). Recombinant *Pf*HRP2 antigen and recombinant *Pf*LDH antigen were purchased from CTK Biotech. (San Diego, CA). Methylene blue (MB) succinimidyl ester was purchased from Biosearch Technologies Inc. (Petaluma, CA). MB-conjugated anti-*Pf*HRP2 IgG was prepared by coupling the amine groups of IgG and active ester group of MB (see Supporting Information) and stored at 4 °C for short-term (≤4 weeks) storage and −20 °C for long-term (> 1 month) storage.

2.2. Preparation and characterization of Pt NPs

Citrate-stabilized Pt NPs were prepared by ascorbic acid reduction as previously reported (Huang et al., 2005) with minor modifications. Briefly, 1.3 mL of 5% H_2PtCl_6-aged solution was added to 68 mL of DI water and stirred at 80 °C. 20 mL of 1% aqueous sodium citrate solution (fresh stock) was slowly dripped into the H_2PtCl_6 solution, and the mixture was stirred for 10 min. 10.7 mL of the 0.1 M ascorbic acid solution was added to the final mixture and stirred for 20 min at 80 °C. Transmission electron microscopy (TEM) images of synthesized Pt NPs were obtained using a JEOL JEM-2200FS electron microscope (Peabody, MA) operated at 200 kV. Dynamic light scattering (DLS) measurements of Pt NPs in DI water (0.5% w/v) were carried out using a Zetasizer Nano (Malvern Instruments, UK).

2.3. Sensor preparation

Electrodes were fabricated using low electrocatalytic indium tin oxide (ITO) on glass and prepared as previously reported (Wang et al., 2013b; Chua et al., 2009; Aziz et al., 2008) with minor modifications. Briefly, ITO-coated glass was cut into 1 cm×2 cm pieces and immersed in a solution of 1:1:5H_2O_2 (30%), NH_4OH (30%) and H_2O (v/v/v) at 70 °C for 90 min to form surface hydroxyl groups, and dried under a stream of purified N_2 gas. Hydroxylated electrodes were then immersed in 2% (v/v) APTES in anhydrous toluene for 1 h at room temperature (RT) to form a silane monolayer, followed by subsequent rinsing in anhydrous toluene, methanol, and DI water. Amine-functionalized electrodes were then incubated in a 10% aqueous glutaraldehyde solution for 30 min at RT, rinsed in DI water, dried using N_2 gas and stored in a desiccator (30% relative humidity) prior to protein immobilization.

2.4. Protein immobilization

70 µL of PBS (pH 7.4) containing 100 µg/mL of anti-*Pf*HRP2 IgM was dispensed onto APTES-glutaraldehyde modified electrodes for 1 h at RT followed by thoroughly rinsing with DI water and drying with N_2 gas. A 10 mM ethanolamine-hydrochloric acid (HCl) solution (pH 8.8) was incubated on the surface for 30 min at RT to deactivate and block the electrode surface from nonspecific binding. The electrodes were then washed twice with PBS and stored at 4 °C for up to 2 weeks prior to experiments. To characterize the electrode surface after protein immobilization, atomic force microscopy (AFM) was performed on the electrode to observe the changes in surface morphology as a result of protein binding (Fig. S2). AFM scans were performed under tapping mode in air using a Cypher S atomic force microscope (Asylum Research, Santa Barbara, CA). Samples were imaged at a constant force of 0.5 N/m, scan rate of 1 Hz and scan size of 2 µm×2 µm. The images were processed and analyzed using Igor Pro software.

2.5. Experimental setup

Cyclic voltammetry and chronocoulometry were performed in a Teflon electrochemical cell at RT using a 620 A potentiostat (CH Instruments, Austin, TX). The cell was comprised of the ITO working electrode, an Ag/AgCl (3 M KCl) reference electrode (CH Instruments), and a Pt counter electrode (CH Instruments). Hydrazine/Pt NP substrates were prepared by combining 100 µL of PBS with 50 mM of hydrazine and 2 µL of DI water containing 0.5% (w/v) Pt NPs. The final concentrations of hydrazine and Pt NPs were 5 mM and 0.001% (w/v) respectively. All substrate solutions were freshly prepared and used immediately for measurements.

2.6. Electrochemical measurements

Samples were prepared by serially diluting *Pf*HRP2 antigen in PBS or unadulterated human saliva. Saliva was collected from healthy volunteers using a passive drool method and used without further purification. 70 µL of spiked PBS or saliva was dispensed onto the ITO electrode and incubated for 30 min at RT followed by washing in PBS. Next, 70 µL of PBS containing 10 µg/mL of MB-labelled anti-*Pf*HRP2 IgG was dispensed onto the electrode, incubated for 30 min at RT and washed twice with PBS. The working electrode was inserted into the electrochemical cell, 1 mL of substrate was injected and incubated for 5 min followed by the application of a 0.05 V bias potential (vs Ag/AgCl).