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A polyaniline based ultrasensitive potentiometric immunosensor for cardiac troponin complex detection

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ABSTRACT

An ultrasensitive immunosensor based on potentiometric ELISA for the detection of a cardiac biomarker, troponin I–T–C (Tn I–T–C) complex, was developed. The sensor fabrication involves typical sandwich ELISA procedures, while the final signal readout was achieved using open circuit potentiometry (OCP). Glassy carbon (GC) working electrodes were first coated with emulsion-polymerized polyaniline/dinonylnaphthalenesulfonic acid (PANI/DNNSA) and the coated surface was utilized as a transducer layer on which sandwich ELISA incubation steps were performed. An enzymatic reaction between o-phenylenediamine (OPD) and hydrogen peroxide (H_2O_2) was catalyzed by horseradish peroxidase (HRP) labeled on the secondary antibodies. The polymer transducer charged state was mediated through electron (e^-) and charge transfers between the transducer and charged species generated by the same enzymatic reaction. Such a change in the polymer transducer led to potential variations against an Ag/AgCl reference electrode as a function of Tn I–T–C complex concentration during incubations. The sequence of OPD and H_2O_2 additions, electrochemical properties of the PANI/DNNSA layer and non-specific binding prevention were all crucial factors for the assay performance. Under optimized conditions, the assay has a low limit of detection (LOD) (<5 pg/mL or 56 fM), a wide dynamic range (>6 orders of magnitude), high repeatability (coefficient of variance $<8\%$ for all concentrations higher than 5 pg/mL) and a short detection time (<10 min).

1. Introduction

Cardiovascular diseases (CVDs), such as acute myocardial infarction (AMI), are currently among the leading causes of human mortality (WHO, 2014). The development of rapid, sensitive and low-cost diagnostic approaches for such diseases is a crucial step towards therapeutic interventions (Yang and Zhou, 2006). At elevated levels in the blood of AMI patients, cardiac biomarkers such as C-reactive protein (CRP), troponin I (cTnI), troponin T (cTnT), myoglobin and the MB isoenzyme of creatine kinase (CK-MB) are major indicators in AMI diagnosis (Ellenius et al., 1997; Panteghini et al., 1999; Hasdai et al., 2003; Qureshi et al., 2012).

Enzyme-linked immunosorbent assay (ELISA), based on specific antibody–antigen interactions, provides the possibility of quantifying a wide range of cardiac biomarkers in biological fluids at good sensitivity and selectivity (Engvall and Perlman, 1971; Voller et al., 1978; Lequin, 2005). A conventional ELISA relies on optical density readouts as chromogenic reactions are triggered by the enzymatic reporters tethered to the secondary antibodies. More recent signal readout methods involve the use of fluorogenic (Levine and Raines, 2011), electrochemiluminescent (Phillips and Abbott, 2008), and real-time PCR (Tahk et al., 2011) reporters.

Electrochemical ELISAs, where the detections are realized by electrochemical readouts rather than optical measurements, have emerged as alternative assays. They exhibit immense potential for increased sensitivity, simplicity of instrumentation, rapidity of testing and low cost compared to optical ELISAs (Piermarinia et al., 2007; Wei et al., 2009; Ricci et al., 2012; Rusling, et al., 2012). Additionally, miniaturization, microfluidics and multiplexed detection can easily be integrated in such systems (Rusling, et al., 2013; Lin et al., 2010; Kirby et al., 2004). Electrochemical ELISAs that leverage amperometric, capacitive and potentiometric measurements were developed in the recent past (Akanda et al., 2011; Bhimji et al., 2013; Purvis et al., 2003; Labib et al., 2009; Tang et al., 2004; Yuan et al., 2004). Potentiometric ELISA is the most straightforward method among those, as no external excitement such as current and voltage is required during the measurement. However, in label-free potentiometric immunosensors, the

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formation of electrical double layer (EDL) may occlude signals derived from biological bindings and lead to lower sensitivity (Tang et al., 2004; Yuan et al., 2004). Such problems can be largely circumvented if the signal results from redox reaction occurring in close proximity to the electrode surface. Polypyrrole-based potentiometric ELISA exploiting this concept were previously reported (Purvis et al., 2003; Laczka et al., 2013). Sandwich ELISA was first performed on the polymer transducer layer to form HRP labeled immune-complexes. With the oxidation state change of polypyrrole layer upon the transfer of electrons and charged species facilitated by HRP catalyzed reaction, the resultant surface potential variation was amplified and used to quantify the biomarkers at low detection limits. Nonetheless, the understanding of this technique is still preliminary and more research is required for it to advance towards clinical applications.

We studied this technique in greater depth in this contribution. A novel polyaniline (PANI) based potentiometric ELISA for the detection of troponin I–T–C (Tn I–T–-C), a complex of the three distinct single-chain troponin subunits, is presented. Since the stability of cTnI, the most commonly used cardiac biomarker, was proved to be significantly higher in such ternary complexes, Tn I–T–C samples are considered as ideal candidates for AMI diagnosis (Filatov et al., 1999). PANI, a conductive polymer widely used in immunoassays (Liu et al., 2000; Cui et al., 2012a; 2012b), was selected as the transducer layer because of its exceptional pH and redox sensitivity, environmental stability and versatility in bioimmobilization compared to other conjugated conductive polymers (Dhand et al., 2011).

The assay was accomplished with a conventional two-electrode open circuit potentiometry (OCP) system, suitable for multiplexing. Drop-casted PANI/DNNSA layer was successfully used as a transducer, which broadens the range of immunosensor applications on different types of substrates. We also observed that the optimization of transducer electroactivity contributed to an increase in sensitivity by multiple orders of magnitude. The blocking step was confirmed to be effective on the transducer layer as the potential response due to non-specific bindings in the blank test was brought down to ca. 2 mV in 1% BSA/PBS environment and 13 mV in neat human serum environment, resulting in high signal to noise (S/N) ratios. To avoid compromised assay sensitivity incurred by the direct reaction between PANI/DNNSA and OPD, a novel two-step sequential addition of OPD and H_2O_2 was proposed to amplify the signal from HRP catalyzed enzymatic reaction. The optimized assay was demonstrated to be ultrasensitive, rapid, highly repeatable and wide in dynamic range. A much more comprehensive understanding of the underlying potentiometric ELISA achieved in this paper could also expand its applications in ion-modulated biosensing systems such as cutting-edge ion sensitive field effect transistor sensor arrays (Shen et al., 2003; Barbaro et al., 2006; Zhang et al., 2015).

2. Materials and methods

2.1. Materials

Hydrogen peroxide (H_2O_2), citric acid, sodium phosphate dibasic (Na_2HPO_4), glutaraldehyde (GA), o-phenylenediamine (OPD) tablets, PBST tablets (0.05% w/v Tween) and horseradish peroxidase (HRP) were all purchased from Sigma Aldrich. Ethanol (EtOH) was purchased from VWR International. Polyaniline/dinonylnaphthalenesulfonic acid (PANI/DNNSA) mixture was obtained from Crosslink Inc. This mixture with high solubility in a variety of organic solvents was synthesized through emulsion polymerization (Kinlen et al., 1998).

Phosphate buffered saline (PBS) solution (Sigma Aldrich) used in this study (pH 7.4) contains 10 mM phosphate, 2.7 mM KCl and 137 mM NaCl. Citric phosphate (CP) buffer (pH 5.0) was prepared by mixing 0.2 M of 25.7 mL Na_2HPO_4 and 0.1 M of 24.3 mL citric acid aqueous solutions.

ELISA grade bovine serum albumin (BSA) (Sigma Aldrich), casein blocking buffer (1% w/v) (Thermo Scientific), skim milk powder (BD Difco) and superblock buffer (Pierce) were used as blocking reagents. Cardiac troponin I (cTnI) free human serum, monoclonal mouse anti-cardiac troponin I (cTnI, MAb 810), human cardiac Tn I–T–C complex (apparent Mw of 39, 29 and 18 kDa for cTnT, cTnI and TnC) and two types of HRP conjugated monoclonal mouse anti-cardiac troponin I (MAb MF4 and MAb 19C7) were all purchased from HyTest Ltd.

2.2. Polymer coating on working electrodes

Glassy carbon (GC) electrodes with 3 mm disc dia. (BASi) were polished with 1 μm and 0.3 μm Al_2O_3 powders followed by cyclic voltammetry (CV) treatment in 0.5 M H_2SO_4 solution to remove any residues and contaminants on electrode surface. The electrodes were then sonicated in Milli-Q deionized water (18 ΩM) and dried with a stream of nitrogen. The freshly prepared GC electrodes were each coated with 8 μL of 1.5 wt% PANI/DNNSA mixture in $CHCl_3$ and dried in an oven at 60 °C for 2 h.

2.3. Ethanol treatment

Ethanol (EtOH) treatment of the PANI/DNNSA layers was used to adjust its DNNSA/PANI doping ratio for better electroactivity. The PANI/DNNSA coated GC electrodes were treated by dipping them into 100% EtOH for 60 s followed by thorough DI-water rinse and nitrogen drying.

2.4. Energy-dispersive X-ray spectroscopy (EDS)

Quantitative chemical analyses were conducted using Oxford INCA PentaFETx3 EDS system (Model 8100) attached to a field emission scanning electron microscopy (FE-SEM). The EDS system provides fully digital image collection, transfer and analysis. Standard calibration was conducted on the EDS system prior spectral collection for accurate quantitative analysis with an estimated error of $< 1\%$. In addition, for accurate relative quantitative chemical analysis, all EDS spectra were collected under identical electron probe conditions. Probe accelerating voltage was selected in order to excite all elements present in the PANI/DNNSA layer on GC electrodes with sufficient statistical EDS signals for accurate analysis.

2.5. Electrochemical analysis

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were performed using a CHI 660d electrochemical workstation (CH Instruments) with a three electrodes system. It consists of a PANI/DNNSA modified GC electrode, with or without EtOH treatment, as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl electrode (BASi) as the reference electrode.

2.6. Preparation of immunosensing layers

The PANI/DNNSA coated GC electrodes, with or without EtOH treatment, were dipped in CP buffer with 2.5 wt% GA crosslinker at room temperature for 1.5 h followed by thorough wash with DI-H_2O. Afterwards, plastic tubes were mounted on the coated side of the electrodes to create a setting that resembles the wells in conventional ELISAs. Standard sandwich ELISA incubation steps