Biosensors and Bioelectronics 54 (2014) 115–121

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Designing label-free electrochemical immunosensors for cytochrome *c* using nanocomposites functionalized screen printed electrodes

Manickam Pandiaraj [a], Niroj Kumar Sethy [b], Kalpana Bhargava [b], Vepa Kameswararao [c], Chandran Karunakaran [a,*]

[a] Biomedical Research Laboratory, Department of Chemistry, VHNSN College (Autonomous), Virudhunagar 626001, Tamil Nadu, India
[b] Peptide and Proteomics Division, DIPAS, DRDO, Delhi 110054, India
[c] Defence Research & Development Establishment, Gwalior 474002, Madhya Pradesh, India

ARTICLE INFO

Article history:
Received 2 August 2013
Received in revised form
12 October 2013
Accepted 21 October 2013
Available online 31 October 2013

Keywords:
Electrochemical immunosensors
Label-free detection
Cytochrome *c*
ELISA
Screen printed electrodes

ABSTRACT

We have designed here a label-free direct electrochemical immunosensor for the detection of cytochrome *c* (cyt *c*), a heme containing metalloprotein using its specific monoclonal antibody. Two nanocomposite-based electrochemical immunosensor platforms were evaluated for the detection of cyt *c*; (i) self-assembled monolayer (SAM) on gold nanoparticles (GNP) in polypyrrole (PPy) grafted screen printed electrodes (SPE) and (ii) carbon nanotubes (CNT) integrated PPy/SPE. The nanotopologies of the modified electrodes were confirmed by scanning electron microscopy. Electrochemical impedance spectroscopy and cyclic voltammetry were employed to monitor the stepwise fabrication of the nanocomposite immunosensor platforms. In the present method, the label-free quantification of cyt *c* is based on the direct electron transfer between Fe (III)/Fe (II)-heme redox active site of cyt *c* selectively bound to anti-cyt *c* nanocomposite modified SPE. GNP/PPy and CNT/PPy nanocomposites promoted the electron transportation through the conductive pore channels. The overall analytical performance of GNP/PPy based immunosensor (detection limit 2 nM; linear range: 2 nM to 150 μM) was better than the anti-cyt *c*/CNT/PPy (detection limit 10 nM; linear range: 10 nM to 50 μM). Further, the measurement of cyt *c* release in cell lysates of cardiomyocytes using the GNP/PPy based immunosensor gave an excellent correlation with standard ELISA.

1. Introduction

Immunoassay is one of the most effective approaches for specific detection of proteins and has been used extensively for clinical diagnoses and biochemical field (Li et al., 2008; Rieger et al., 2009). The conventional enzyme linked immunosorbent assay (ELISA) is an important, commercial immunoassay widely employed for protein detection (Lilja et al., 2008; Williams et al., 2007). However, ELISA is an optical approach, has limitations in colored samples analysis, experiment time, sample size, and hence difficult to employ as point-of-care testing (Walt, 2005; Wei et al., 2010; Huang et al., 2010). In this context, the development of electrochemical immunosensors as an alternative to the conventional immunoassay systems draws more attention in a wide range of uses, especially for determination of clinically important analytes. Electrochemical immunosensors can provide a precise and real-time measurement of protein biomarkers owing to their unique combination of exquisite specific antigen–antibody interaction and sensitive electrochemical transduction (Liu et al., 2007; Viswanathan et al., 2009; Ricci et al., 2012). Since most of the proteins cannot intrinsically act as redox partners in an electrochemical reaction, the electrochemical protein immunosensors involve the use of some external mediators or labeling either antigen or antibody to achieve electron-transfer (Li et al., 2011; Prabhulkar et al., 2009), all of which lead to an inconvenient, time consuming process. Moreover, the labeling with various agents might influence the antigen–antibody binding efficiency (Qiu et al., 2009). Thus, novel approaches for constructing direct and label-free electrochemical immunosensor alternative to conventional immunosensors are very desirable. The label-free direct electrochemical immunosensors have recently emerged as a novel assay to detect proteins containing prosthetic groups with reversible redox-activity (Okuno et al., 2007; Liu and Gooding, 2009; Lin et al., 2012; Wei et al., 2012; Fan et al., 2013). Suprun and his research team have demonstrated a direct immunosensor for the detection of myoglobin (Mb), a metalloprotein containing electroactive heme (Fe (III)) as prosthetic group using its reversible electron transfer of Mb–Fe (III)/Mb–Fe(II) heme with the modified electrode (Suprun et al., 2010; Shumyantseva et al., 2009; Suprun et al., 2011).

Cytochrome *c* (cyt *c*) a heme (Fe (III)) containing, biologically important mitochondrial redox protein, plays an important

* Corresponding author. Tel.: +91 4562 280154; fax: +91 4562 281338.
E-mail addresses: ckaru2000@gmail.com, ckaru2000@yahoo.com (C. Karunakaran).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.10.030

physiological role in oxidative phosphorylation (Ow et al., 2008; Hüttemann et al., 2011) and also as an electron carrier in the mitochondrial intermembrane space between cyt *c* reductase (complex III) and cyt *c* oxidase (complex IV). However, cyt *c* can be translocated out from mitochondria to cytosol under various pathological conditions (Radhakrishnan et al., 2007), triggering the activation of caspases and subsequent apoptotic cell death (Wetzel et al., 1998; Hengartner, 2000). Moreover, cyt *c* release also have been identified in circumstances that can injure mitochondria, such as acute myocardial infarction, chemotherapy, a debilitating brain injury, and various neurological diseases (Pullerits et al., 2005; Radhakrishnan et al., 2007). So, the quantitative detection of trace amount of cyt *c* release in biological samples is of great importance as preclinical diagnosis.

In the literature, the direct determination of cyt *c* has been carried out based on the electrostatic interaction between the cyt *c* (positive charge) and the recognition elements (negative charge) (Liu and Wei, 2008; Zhao et al., 2008). Cyt *c* oxidase (CcO) based cyt *c* biosensors have been also reported (Ashe et al., 2007; Batra et al., 2013). However, these methods either lack specificity or measure only the non-apoptotic form of cyt *c* (Fe (II)). To overcome this, we have recently reported cyt *c* reductase (CcR) based biosensor for the quantification of mitochondrial cyt *c* release during carcinoma A549 cell apoptosis (Pandiaraj et al., 2013). In this paper, we report the design and evaluation of a new electrochemical immunosensor assay for the measurement of cyt *c* release by immobilizing its specific monoclonal antibody onto the surface of nanocomposite modified screen printed electrodes (SPE). For label-free detection of cyt *c*, the specific antibody immobilized electrode was allowed to bind with cyt *c* and monitor the difference in the cyclic voltammetric current response before and after the immunological binding. The direct electron communication between the heme active center of cyt *c* and the electrode surface has been used as mechanism for cyt *c* detection, without the participation of any mediators or other enzyme labeled secondary antibody.

Although label-free immunosensors can provide a sensitive detection, it still requires further modification to promote the electron transfer of metalloprotein due to their slow electron transfer at bare electrodes. High surface area nanoparticle modified electrodes offer unprecedented opportunities for the design of highly stable and sensitive immunosensors to detect proteins (Rusling et al., 2009; Munge et al., 2009; Sun et al., 2011). These nanomaterial architectures act as electron-conduction tunnels and promoted the electrical communication between redox sites of protein and the sensing surface. Especially, the nanocomposite of gold nanoparticles (GNP) combined with conducting polymer to provide a stable immobilization of antibodies retaining their bioactivity towards antigen binding is a major advantage for the preparation of immunosensors (Qu et al., 2009; Liu et al., 2011). Further, the incorporation of carbon nanotubes (CNT) into composite electrode matrices have also attracted much interest for the design of electrochemical immunosensors with improved analytical performances (Agui et al., 2008; Viswanathan et al., 2012). So, both the GNP and CNT platforms employed here to enhance the direct electron transfer between the cyt *c* and the electrode surface, thus allowing electrochemical immunosensing to be performed with high sensitivity.

2. Materials and methods

2.1. Reagents and instrumentation

Cyt *c* from bovine heart, bovine serum albumin (BSA), hydrogen peroxide (H_2O_2), glutaraldehyde, sodium hydrogen phosphate, disodium hydrogen phosphate, chloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$), sodium nitrate, ethidium bromide, acridine orange, and Bradford reagent were purchased from Sigma (St. Louis, MO, USA). Cyt *c* monoclonal antibodies were purchased from Invitrogen, CA, USA. Single walled CNT were purchased from Carbon solutions Inc., CA, USA. Dulbecco's modified Eagle's Medium (DMEM), and Trypsin 1X solution were purchased from HiMedia, India. Rat cardiomyocytes cell line H9c2 was obtained from American type culture collection (ATCC), USA. All the solutions were prepared with doubly distilled water.

A planar screen-printed electrode (SPE) consisting of a carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode (Zensor R&D, Taiwan) was used for designing of electrochemical immunosensors. All cyclic voltammetric measurements were carried out with a hand held CHI 1200B electrochemical workstation (CH Instruments, USA). Electrochemical impedance measurements were done on a VMP3 – Modular 16 Channels Potentiostat/Galvanostat/EIS from Bio-logic science instruments, France. The surface morphologies of the modified electrodes were characterized by scanning electron microscope (SEM) and energy dispersive X-ray analysis (EDX) (FEI Co., Netherlands). The phase contrast images were acquired by using an Olympus-1 × 71 inverted microscope (Olympus Co., USA).

2.2. Construction of anti-cyt c/SAM/GNP/PPy/SPE immunosensor

The first step in the fabrication of anti-cyt *c*/SAM/GNP/PPy/SPE immunosensor is electropolymerization of pyrrole to form conducting polypyrrole (PPy) matrix onto the working electrode of SPE. Prior to the electropolymerization, the SPE was pretreated to remove organic ink constituents or contaminants and to increase surface roughness, functionalities. The pretreatment of the SPE was carried out by cycling the potential between −0.5 and 1.0 V at a scan rate of 100 mV/s in 1 M H_2SO_4 for 10 complete cycles (Nassef et al., 2008). Then, the SPE was washed with Milli-Q water and dried at room temperature. After pretreatment, the grafting of PPy was performed by electropolymerization of pyrrole on working electrode of SPE by following our previous procedure (Rajesh et al., 2010). The obtained electrode was now represented as PPy/SPE.

Electrochemical deposition of GNP onto the PPy/SPE is the second step in the fabrication of immunosensor. Cyclic voltammetry was employed for the electrodeposition of GNP at room temperature. A negative sweep of the electrode potential from 0.9 V to 0 V at a scan rate of 100 mV/s was carried out in 0.25 mM $HAuCl_4 \cdot 3H_2O$ solution containing N_2-saturated 0.1 M $NaNO_3$ as electrolyte (Hezard et al., 2012). The optimum of five cycles was used for homogenous electrodeposition of GNP onto the PPy/SPE. The self-assembled monolayer (SAM) formation on nanostructured GNP/PPy/SPE was further carried out by incubating the working electrode surface with 5 μL of 1 mM cysteine solution for 1 h at room temperature. After that, the SAM modified GNP/PPy/SPE was rinsed twice with Milli-Q water.

The final step of the immunosensor fabrication is stable immobilization of monoclonal cyt *c* antibody (anti-cyt *c*) onto the SAM/GNP/PPy/SPE. For this, a freshly prepared anti-cyt *c* solution (10 μL) was incubated onto the SAM/GNP/PPy/SPE using glutaraldehyde as a crosslinking agent. Then, the assembled immunosensor (anti-cyt *c*/SAM/GNP/PPy/SPE) was thoroughly rinsed with PBS to remove any unbound anti-cyt *c* and stored at 4 °C when not in use.

2.3. Construction of anti-cyt c/CNT/PPy/SPE immunosensor

The immunosensor platform features here a CNT tailored PPy/SPE instead of GNP electrodeposited PPy/SPE. The incorporation of