Sensors and Actuators B 196 (2014) 123–132

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

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

Protein-responsive polymers for point-of-care detection of cardiac biomarker

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ARTICLE INFO

Article history:
Received 13 November 2013
Received in revised form
25 December 2013
Accepted 10 January 2014
Available online 7 February 2014

Keywords:
Biomimetic antibodies, Screen-printed electrodes
Molecular imprinting
Electropolymerization
Myoglobin
Biosensor

ABSTRACT

This work describes a novel use for the polymeric film, poly(o-aminophenol) (PAP) that was made responsive to a specific protein. This was achieved through templated electropolymerization of aminophenol (AP) in the presence of protein. The procedure involved adsorbing protein on the electrode surface and thereafter electroploymerizing the aminophenol. Proteins embedded at the outer surface of the polymeric film were digested by proteinase K and then washed away thereby creating vacant sites. The capacity of the template film to specifically rebind protein was tested with myoglobin (Myo), a cardiac biomarker for ischemia. The films acted as biomimetic artificial antibodies and were produced on a gold (Au) screen printed electrode (SPE), as a step towards disposable sensors to enable point-of-care applications.

Raman spectroscopy was used to follow the surface modification of the Au-SPE. The ability of the material to rebind Myo was measured by electrochemical techniques, namely electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV). The devices displayed linear responses to Myo in EIS and SWV assays down to 4.0 and 3.5 μg/mL, respectively, with detection limits of 1.5 and 0.8 μg/mL. Good selectivity was observed in the presence of troponin T (TnT) and creatine kinase (CKMB) in SWV assays, and accurate results were obtained in applications to spiked serum. The sensor described in this work is a potential tool for screening Myo in point-of-care due to the simplicity of fabrication, disposability, short time response, low cost, good sensitivity and selectivity.

1. Introduction

Cardiovascular diseases affect the heart and surrounding blood vessels, and manifest many forms, such as high blood pressure, coronary artery disease, valvular heart disease, stroke, or rheumatic heart disease. These are the largest single cause of death in the EU, accounting 42% deaths in 2010 [1]. Following cardiovascular biomarkers is a key tool in the fight against cardiovascular diseases, aimed at preserving the patient's life and delivering effective therapies and a successful prognosis for the disease [2–4].

Acute events in cardiovascular diseases are here of special concern, and have been successfully diagnosed by monitoring several well-known biomarkers, such as Creatine kinase isoenzyme (CKMB) [5,6], cardiac troponin I or T (Tn) [5,7], brain natriuretic peptide (BNP) [8] and Myoglobin [3,4]. Timely measurements of these biomarkers demands access to low-cost devices, providing rapid responses and capable of operating in point-of-care.

Current techniques used to determine cardiovascular biomarkers rely on immune/antigen reactions, in a wide range of approaches that include counter-immunoelectrophoresis, latex agglutination quantitative microcomplement fixation assay, and radioimmunoassay [4,9,10]. These rely on the great specificity of an antibody towards its antigen, a condition that ensures an easy handling of complex blood samples. However, most of these are not portable and require procedures that can only be made in a clinical laboratory, which is clearly inappropriate for point-of-care applications. The overall procedure may also take much longer than desired in acute events, reaching 2 h. Alternative methods to immunoassays use highly sophisticated chromatographic or electrophoretic procedures [11–13], which are expensive and again unsuitable to carry out analysis in point-of care.

It is therefore important to look for other ways of monitoring biomarkers in cardiovascular diseases, bringing significant long-term economic, health and social benefits to our society.

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http://dx.doi.org/10.1016/j.snb.2014.01.038

(Bio)Sensors could be an alternative, because they allow rapid diagnosis in point-of-care, providing reduced response times and leading to better outcomes of the disease, most especially in acute events [2,14]. The receptor element is at the core of the device selectivity, an essential condition for detecting proteins that circulate in the blood at quite low concentrations.

Natural receptors namely antibodies, nucleic acids, and enzymes, have been widely used in biosensors for targeting cardiac biomarkers, leading to electrochemical, optical, mass or magnetic changes [2]. In general, natural receptors offer high selectivity towards a target analyte, but show decreased chemical stability, and are rather expensive. Thus, biomimetic materials have been sought for many years as synthetic receptors.

Molecularly imprinted polymers (MIPs) are among these biomimetic materials and act as plastic antibodies in chemical sensors. In general, MIPs are obtained by 3-D (bulk) or 2-D (surface) imprinting of the complete molecule or parts of it (epitopes) in a rigid polymeric matrix of either organic (vinyl functional derivatives) or inorganic (silica derivatives) materials. The template molecule is subsequently removed and the resulting polymeric network keeps the ability to rebind the template because of the steric and electronic characteristics of the resulting cavities [15,16]. Molecular imprinting technology creates materials whose binding specificity is similar to that of antibody–antigen interactions [17,18]. These materials offer unique opportunities in terms of reversible inclusion of a bioanalyte [19], mechanical/chemical stability, low price potentially and compatibility with mass manufactures [18,20–22].

While the imprinting of small molecules has been successfully achieved, protein imprinting is still a challenge [23]. The restricted protein mobility within the polymeric network and the weak efficiency/reversibility of binding are major technical hitches. 2-D imprinting seems more reliable in this case, because the binding sites are exposed at the surface allowing easy access/removal of the proteins to/from these. However, in surface imprinting the number of binding sites is rather limited compared to bulk, by the small area available for (re)binding. Thus, a suitable MIP design must be selected.

Few strategies of protein surface imprinting in cardiac biomarker sensing context have been employed so far [23–29], involving surface imprinting procedures on gold [24,28–30], silica [25,26], or carbon [27], while employing different approaches for assembling the polymeric network. Among these, electropolymerization seems to be the simplest approach towards protein imprinting that seems to have been developed first by Panasyuk et al. for a small target analyte [31], and applied later to proteins by Cai et al. [32].

Electropolymerization is typically conducted by leaving the template within the monomer in solution and by applying a suitable potential to enable the formation of a thin film on the electrode surface. Electropolymerization is thus a very simple procedure, enabling the use of different conductive materials of different shape/size, and a close control of the polymer thickness by adjusting the electrochemical conditions under which the polymer is formed [33,34]. However, many of the protein structures, are typically nm in size, are thus irreversibly entrapped inside the polymeric network and only few are at the outer surface of the newly formed film. The only specific work in this context is that of Karimian et al. most recently published [30]. The authors report a successful electropolymerized imprinted film for troponin, by mixing the cardiac biomarker in the solution with *o*-phenylenediamine, acting as the monomeric unit of the film.

An alternative approach to this procedure, never tested before, as far as we know, would be first adsorbing the proteins to the electrode surface (possibly as multilayers) and to control the thickness of the polymer film, to ensure that most of these proteins are not irreversibly covered and so can be removed. A high density of imprinted cavities is thus expected. Thus, this new concept of electropolymerization for imprinting a protein cardiac biomarker is here described, using Myo as target protein. Myo is a heme protein responsible for the transport of oxygen within cardiac and skeletal muscle cells [35,36] that is released early in the blood in the course of cardiac muscle damage. The measurement of Myo levels within the first few hours after symptom onset in acute events is crucial to diagnosis of cardiac injury. Typical concentrations range from 95 to 472 μg/L [35,37–39].

Overall, Myo imprinted films (MI) were prepared by electropolymerizing *o*-aminophenol around a protein layer previously absorbed to gold. The protein structures located at the outer surface of the film were removed by protease action. A non-imprinted (NI) film was also synthesized in the absence of template to act as control. The resulting biosensor was evaluated by several electrochemical techniques such as CV, EIS and SWV techniques and further applied to the analysis of biological sample.

2. Experimental

2.1. Apparatus

The electrochemical measurements were conducted with a potentiostat/galvanostat from Metrohm Autolab and a PGSTAT302N, equipped with a FRA module and controlled by Nova software. The Au-SPEs were purchased to DropSens, and have working and counter electrodes made of gold with a reference electrode and electrical contacts made of silver. The diameter of the working electrode was 4 mm. The SPEs were connected to a switch box, also from DropSens (DRP-DSC), allowing their interface with the potentiostat/galvanostat.

Fourier transform infrared spectroscopy (FTIR) measurements were performed using a Thermo Scientific Smart iTR Nicolet iS10, coupled to a SAGA smart accessory, also from Thermo Scientific. Raman measurements were performed using a Thermo Scientific DXR Raman microscope system with a 100 mW 532 nm excitation laser, and spectra were made for 5 mW power and 50 μm pinhole aperture.

2.2. Reagents

All chemicals were of analytical grade and water was de-ionized or ultrapure Milli-Q laboratory grade. Potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$) and potassium hexacyanoferrate II ($K_4[Fe(CN)_6]$) trihydrate, and sodium acetate anhydrous, were obtained from Riedel-deHaen; bovine serum albumin (BSA), urea, Myo, Troponin T (TnT), creatine kinase (CKMB), proteinase K and sodium chloride from Fluka; AP 99%, 2-(*N*-Morpholino)ethanesulphonic acid monohydrate 98% (MES) from Alfa Aesar; and potassium chloride (KCl) from Merck.

2.3. Solutions

Stock solutions for calibration curve of 5.0×10^{-6} mol/L Myo were prepared in MES buffer (1.0×10^{-2} mol/L, pH 5.0). Less concentrated standards were obtained by accurate dilution of the previous solution, in the same buffer. Electrochemical assays were performed with 5.0×10^{-3} mol/L $K_3[Fe(CN)_6]$ and 5.0×10^{-3} mol/L $K_4[Fe(CN)_6]$, prepared in MES 1.0×10^{-3} mol/L, pH 5.0. The selectivity study used 4.0 μg/mL Myo solutions prepared in buffer and solutions of possible interfering species,