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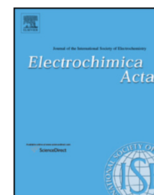

Electrochemical biosensor based on biomimetic material for myoglobin detection

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ABSTRACT

A novel reusable molecularly imprinted polymer (MIP) assembled on a polymeric layer of carboxylated poly(vinyl chloride) (PVC—COOH) for myoglobin (Myo) detection was developed. This polymer was casted on the gold working area of a screen printed electrode (Au-SPE), creating a novel disposable device relying on plastic antibodies. Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and Fourier transform infrared spectroscopy (FTIR) studies confirmed the surface modification.

The MIP/Au-SPE devices displayed a linear behaviour in EIS from 0.852 to 4.26 $\mu g\,mL^{-1}$, of positive slope 6.50 ± 1.48 ($k\Omega\,mL\,\mu g^{-1}$). The limit of detection was 2.25 $\mu g\,mL^{-1}$. Square wave voltammetric (SWV) assays were made in parallel and showed linear responses between 1.1 and 2.98 $\mu g\,mL^{-1}$. A current decrease was observed against Myo concentration, producing average slopes of -0.28 ± 0.038 $\mu A\,mL\,\mu g^{-1}$. MIP/Au-SPE also showed good results in terms of selectivity. The error% found for each interfering species were 7% for troponin T (TnT), 11% for bovine serum albumin (BSA) and 2% for creatine kinase MB (CKMB), respectively. Overall, the technical modification over the Au-SPE was found a suitable approach for screening Myo in biological fluids.

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1. Introduction

The selective detection/quantification of proteins, as biomarkers of diseases, environmental monitoring, and food control has been a subject of great interest in the last decade [1]. Biosensors are emerged in this context as primary tools. They offer many advantages such as low cost, versatility, good sensitivity, selectivity, and reliability [2,3]. Biosensors based on screen-printed electrodes (SPE) are especially attractive for point-of-care/on-site monitoring because of their small-size and portability. The easy of production and low-cost materials involved in the screen-printing technology have lead to the massive production of such devices [4]. SPEs are also disposable devices, which is an important feature for clinical applications.

In general, biosensors are capable of transforming their interaction with specific analytes into an electronic signal. At the core of this feature is a biorecognition element that is expected to establish exceptionally selective interactions with a target analyte. Biorecognition elements include a wide range of macromolecules, such as enzymes, receptors, ion channel proteins, nucleic acids, aptamers, peptides or antibodies. These were first from natural origin but the advances of nanotechnology have come up with many synthetic versions of these elements [5–7].

The great selectivity of the antibodies has always been a great advantage for biosensors [8,10,11], a feature that justifies that most biosensors described in the literature for Myo detection were immunosensing devices [8,9]. All these display good features in terms of linear range and selectivity. However, natural antibodies are also very expensive material, usually produced by an animal dependent process, and unable to be reused after their first contact with the target template.

Thus, the great cost of these biorecognition elements in parallel to their small stability, has led researchers to the design new materials capable of mimicking the response of antibodies. This has been tried out for several decades, with the most successful approaches using molecular imprint technology [12,7,13].

This is a low-cost and simple approach for designing selective binding sites in polymeric matrices using the target molecule as template. This technology offers great promise for the development of stable artificial bio-sensing elements [14].

Several techniques have been employed in molecular imprinting, differing mainly from the type of interaction between the

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functional monomer and the template prior to polymerization. This may be covalent, non-covalent or semi-covalent nature [15]. Non-covalent imprinting is by far the most popular strategy [16] due to the easy preparation and the wide range of monomers available for this process. The template may also be introduced in the system differently: it may stand free or be attached to a surface, thus creating, respectively, 3D or 2D imprinting environments. The former one is also called surface imprinting and seems to improve protein binding kinetics [17], overcoming some difficulties to mass transfer and protein removal in imprinted matrices [13].

Thus, combining surface imprinting with SPE technology seems an advantageous approach to create new disposable and low cost biomedical devices. This concept has been applied here by assembling the MIP directly on the SPE working area, having Myo as target protein. Myo can be detected in biological fluids such as serum and urine. Myo cut-off values in serum range from 100 to 200 ng mL^{-1} [9,18,19]. In acute myocardial infarction (AMI) condition, Myo maximum levels are variable; concentrations of 420–600 ng mL^{-1} have been found in previous studies [18]. After 4–50 h of an AMI episode, Myo is also expected in urine [20]. These levels are also rather variable and may reach 450 mg mL^{-1} [20,21].

So far, different strategies for Myo detection and quantification based on surface imprinting were described in the literature, including sol–gel methods merged with self assembling monolayer for potentiometric transduction [6,22], silicon surface grafting [13] and thin film/micro contact imprinting mass with spectrometry-based profiling system [23], none of these coupling these two simple concepts of MIP receptors and SPE technology.

Several techniques have been described in the literature for Myo quantification [24–28]. Electro-analytical techniques [9,19,29–33] have concerned considerable attention due to its sensitive, non-destructive, and rapid electrochemical sensing method.

This research paper describes a new disposable biomedical device for monitoring Myo in point-of-care, designed by coating the conductive working area of a SPE with a PVC—COOH film and assembling the MIP on top of it. For this purpose, Myo was bound to the —COOH groups after their activation and the MIP designed by a polymeric matrix, amide in nature. This matrix had non-charged amide groups, polymerized in aqueous environment and was biocompatible to protein targets. Finally, the template was removed by chemical treatment as suggested in [34]. The several steps of surface modification and the analytical performance of the resulting device were controlled by electrochemical techniques and FTIR studies. The MIP/Au-SPE characteristic towards the quantitative estimation of the Myo concentration was investigated by EIS and SWV, $[Fe(CN)_6]^{3-}/^{4-}$ was used as a redox probe. The biosensor showed good electrochemical features in terms of response time, sensitivity and selectivity. This biodevice shows to be a powerful tool for screening Myo in patients with ischaemic episodes.

2. Experimental

2.1. Apparatus

The electrochemical measurements were carried out using a Metrohm-autolab potentiostat/galvanostat Autolab PGSTAT302N interfaced to computer. The SPEs were purchased from Dropsens, Spain (DRP-C220AT), and had a working gold electrode of 4 mm and a silver flat material acting as pseudoreference. Infrared spectra were collected by a Nicolet 67 0 Fourier transform infrared spectroscopy (FTIR) spectrometer. Measurements were conducted in ATR (Attenuated Total Reflectance) mode by a Nicolet ATR sampling accessory of diamond contact crystal. When necessary, the pH was measured by a Crison CWL/S7 combined glass electrode connected to a decimilivoltammeter Crison, pH metre, GLP 22.

2.2. Reagents

All chemicals were of analytical grade and de-ionized water (conductivity <0.1 μS cm^{-1}) was employed. The following reagents were employed: Myo from cardiac muscle (Fluka); PVC—COOH (Fluka); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Fluka); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, Fluka); oxalic acid (Oac, Fluka); *N*,*N*-methylenebisacrylamide (NNMBA, Fluka); albumin, bovine serum (BSA, Fluka); creatine kinase MB (CKMB, Fluka); ammonium persulphate (APS, Fluka); potassium chloride (KCl, Panreac); (hydroxymethyl)aminomethane (Tris, Panreac); potassium dihydrogen phosphate (KH_2PO_4, Penreac); acrylamide (AAM, Fisher Bioreagents); *N*-hydroxysuccinimide (NHS, Acros); tetrahydrofuran (THF, Riedel-deHäen); potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$; Riedel-deHäen); sodium chloride (NaCl, Riedel-deHäen); sodium phosphate dibasic dehydrate ($Na_2HPO_4{\cdot}2H_2O$, Riedel-deHäen); potassium hexacyanoferrate (II) ($[Fe(CN)_6]^{4-}$) trihydrate (Riedel-deHäen); and sodium hydrogen phosphate (Na_2HPO_4) dihydrate (Riedel-deHäen).

2.3. Solutions

Buffer solutions were 1.0×10^{-1} mol dm^{-3} PBS, 1.0×10^{-1} mol dm^{-3} Tris or 1.0×10^{-2} mol dm^{-3} HEPES. The pH of the buffer solution was adjusted to the desired value by adding suitable volumes of either concentrated hydrochloric acid or saturated sodium hydroxide solution freshly prepared.

Stock standard solutions of Myo were 5.0×10^{-7} mol dm^{-3} and prepared in buffer. Less concentrated solutions were prepared by accurate dilution of the previous solution in the same buffer.

2.4. Synthesis of biomimetic materials

The working area of the SPE (gold) was cleaned by washing three times with ethanol. The PVC—COOH layer was obtained by casting on the about 5 μdm^{-3} of a solution of 0.021 g of PVC—COOH dissolved in 2.5 ml THF. The complete dissolution of the polymer was achieved by magnetic stirring inside a fume hood. About 5 μdm^{-3} of this solution was casted. This solution was let dry for 4 h, at room temperature. To activate the —COOH groups, the dry polymer layer was incubated for 3 h in an aqueous solution of 50 mmol dm^{-3} EDAC and 25 mol dm^{-3} NHS. The electrode was then rinsed thoroughly with distilled water to remove un-reacted species.

The imprinting stage started by incubating the activated polymer layer in 5×10^{-6} mol dm^{-3} Myo solution, prepared in 0.1 mol dm^{-3} PBS buffer of pH 7.4. The incubation time was set to 4 h, at 4 °C. After, the electrode was washed twice with PBS buffer (pH 7.4) to remove any exceeding Myo that remained unbound. The sensor was then incubated in 0.5 mol dm^{-3} Tris for 30 min, in order to block any activated group left over in the surface. Several washes with deionised water followed this procedure. The polymerization stage started by adding 1 ml of a solution containing 1 mol dm^{-3} AAM and 0.07 mol dm^{-3} NNMBA, in PBS buffer, pH 7.4, and 1 ml of 0.06 mol dm^{-3} APS solution, in the same buffer. This polymerization was carried out at room temperature for 5 h, after which the sensor was thoroughly washed with deionised water several times.

The sites displaying complementary features to Myo were obtained by extracting the template from the polymer. Strong acids have the ability to break the protein covalent bonds. For this purpose, 0.5 mol dm^{-3} solution of Oac was added to the sensory surface. This was done at room temperature, for 3 h. The MIP was finally washed and conditioned in phosphate buffer, pH 7.4, in order to increase the pH and remove the peptide fractions produced by Oac treatment. The MIP-SPE was ready to use after washing thoroughly with water.

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