Biosensors and Bioelectronics 56 (2014) 217–222

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Novel sensory surface for creatine kinase electrochemical detection

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ARTICLE INFO

Article history:
Received 4 October 2013
Received in revised form
22 December 2013
Accepted 23 December 2013
Available online 16 January 2014

Keywords:
Creatine kinase isoenzyme
CK-MB
Self-assembled monolayer
Screen-printed electrodes
Senso

ABSTRACT

This work describes a novel concept of biosensor for quantifying enzymes, where the substrate is immobilized directly over the working area of a screen printed electrode (Au-SPE). This concept is applied here to creatine kinase isoenzyme (CK-MB), a cardiac biomarker in ischemic conditions. It acts as a phospho-transferase on creatine (Crea), requiring the presence of phosphate. So, the phosphorylated form of creatine (Pcrea) was immobilized on the Au/SPE previously aminated with cysteamine (Cys) by self-assembling monolayer technique. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) studies were used to follow the chemical modifications in the Au-SPE.

Since Pcrea is an electroactive species at low potential, its consumption over the platform by the enzyme changed the electrical response of the biosensor. So, CK-MB determination has been achieved in mediator free-conditions due the redox proprieties of the Pcrea. The analytical features of the resulting biosensor were studied by square wave voltammetry (SWV). The limit of detection was 0.11 μg/mL and the slope was $-0.029(\pm 0.0035)\ \mu A \times mL/\mu g$. The interference effect of troponin T (TnT), bovine serum albumin (BSA) and myoglobin (Myo) in the performance of the sensor was tested and good selectivity was observed.

The biosensor was successfully applied to biological fluids, showing good stability at room temperature and excellent sensitivity and selectivity. This new concept of biosensor is especially useful for point of care (POC) applications, due to the low cost and small size of the final device.

1. Introduction

Heart failure has gained special attention and impact in the society and health systems due to its prevalence, incidence and mortality (Rosamond et al., 2007). Several millions of patients experience acute coronary syndrome (ACS), but only 10% have acute myocardial infarction (AMI) (Collinson, 1999). The criteria for an accurate diagnosis of myocardial infarction require the evaluation of (i) typical symptoms, (ii) biomarkers level and (iii) electrocardiogram (ECG) pattern. The electrocardiographic testing is highly specific for AMI when the typical symptoms are observed, but only about 50% sensitive for AMI in initial examination.

Thus, screening programs and early diagnostic relying on non- or minimally-invasive methods with high sensitivity and specificity, easily available and cost effective are highly desired. These may be reached by the early detection of biomarker proteins that are found early in the body and that have predictive value for the intended disease (Battistoni et al., 2012; Kugelmass et al., 2004). CK-MB is among cardiac biomarkers of clinical interest (Aldous, 2013; Fellahi et al., 2011; Hudson et al., 1999). The muscle isoenzyme of CK is highly specific to cardiac injury and its levels in serum became abnormal within 4–6 h after onset of AMI, peaking at 18–24 h in the range of 39–185 ng/mL (Slovacek et al., 1995; Yang and Zhou, 2006).

Immunoassays are the preferred methods for tracking clinical biomarkers, offering advantages in terms selectivity and multi-analyte testing capability. Several immunoassay methods based on chemiluminescence (Bankson et al., 1989; McBride et al., 1988, 1991; Piran et al., 1987) detection and enzyme-linked immunosorbent assay (ELISA) (Chiu et al., 1999; Curley et al., 1989; Torabi et al., 2007) have been reported so far for CK-MB analysis. These are however expensive, require specific handling conditions, may take more than desired to provide a response and cannot be reused for further testing (Cortese and Ritzlin, 1991; Hoshino et al., 2009; Kato and Shimizu, 1986; Kato et al., 1985; McBride et al., 1991). In addition, the production of antibodies is dependent on animal-host.

Enzymes may turn out an alternative to the use of anti body/antigen as selective biorecognition elements for biosensor development. Enzymes may detect various substrates, which are converted into different molecules, entitled products. Some research works have been described in the literature with

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http://dx.doi.org/10.1016/j.bios.2013.12.052

enzymatic biosensor for to determine CK-MB. Davis et al. (1986) reported an electrochemical assay for the detection of ATP and creatine kinase coupled through hexokinase to an amperometric glucose enzyme electrode. Fonong (1989) described an enzymatic assay for serum creatine kinase-MB isoenzyme determination by an immunoinhibition-amperometric technique. Two enzymes in the scheme, glycerokinase and glycerol-3-phosphate oxidase, were co-immobilized on collagen membrane at the surface of a platinum electrode polarized at 700 mV.

Overall, an enzymatic-based biosensor is a challenging and promising assay due to the high specificity and selectivity of enzymes. However, these biocatalysts are limited in the number of reactions they are evolved to catalyze, which limit the range of application. Additionally, enzymatic electrodes are instable in non-physiological conditions and high temperatures, thus contributing for restricted operating conditions. The immobilization of enzymes is also a crucial factor for the efficiency of the biosensor in terms of selectivity, reproducibility, sensitivity, response time and stability. It is vital that the immobilized biomolecule preserves its conformation and biological activity after covalent surface attachment (Sassolas et al., 2012), since changes affect directly the performance of the biosensor.

Different immobilization approaches of enzymes on the transducer surface have been reported so far, including (i) entrapment (Bossi et al., 2002; Sohail and Adeloju, 2008), (ii) adsorption (Mu and Xue, 1996), (iii) covalent immobilization and cross linking (Lin et al., 2009; Portaccio et al., 2007), and (iv) affinity and combination of these (Andreescu et al., 2003; Andreescu and Marty, 2006). The choice of the most suitable technique is correlated with the enzyme nature, the transducer and the associated transduction method. Reproducibility, cost and difficulty of the immobilization process need to be considered (Sassolas et al., 2012). Sensitivity decreases if immobilization causes enzyme denaturation or conformational changes or if the enzyme has been modified, especially on its active site. Oriented immobilization on transducer surfaces allow better results in terms of sensitivity due the correct exposure of the active sites to the solution phase. Many immobilization techniques involve random distribution or poor orientation of enzyme molecules inducing a partial or a total loss of activity due to enzyme denaturation of the active site or hindered substrate accessibility (Sassolas et al., 2012). Self-assemble monolayer covalent immobilization reduces the number of random orientations, generates uniform, reproducible and stable structures with high coverage (Sassolas et al., 2012).

Overall, enzymes are attached to a receptor surface as a selective and sensitive way to measure any substrate/product directly or indirectly involved in the catalytic reaction. The present case, however, is devoted to the determination of the enzyme, not substrate or product, because the enzyme is the one acting as cardiac biomarker. So, despite the major achievements within enzymatic biosensors, the present work must use a completely different and novel approach.

In a complementary approach to that developed in enzyme biosensors, the substrate is immobilized here in the biosensor, in a very simple and completely new approach never presented before. In order to develop a POC biosensing device, this concept was tested over the gold surface of an Au-SPE modified by self-assembly. Pcrea was covalently linked over the Au-SPE film, previously treated with Cys. The resulting biosensor was evaluated by several electrochemical techniques and further applied to the analysis of biological samples.

2. Experimental section

2.1. Apparatus

Electrochemical measurements were conducted in a potentiostat/galvanostat from Metrohm Autolab/PGSTAT302N, impedimetric module and controlled by Nova software. Au-SPEs were purchased from DropSens (DRP-C220AT), having working and counter electrodes made of gold and reference electrode and electrical contacts made of silver. The diameter of the working electrode (WE) was 4 mm. For electrochemical assays the SPEs were placed in a switch box from DropSens, interfacing the electrical contacts of the Au-SPE with the electrical connections of the potentiostat/galvanostat.

2.2. Reagents

All chemicals were of analytical grade and de-ionized water (conductivity < 0.1 μS/cm) was employed. Potassium hexacyanoferrate II-3-hydrate ($K_4[Fe(CN)_6].{}_3H_2O$) and potassium hexacyanoferrate III ($K_3[Fe(CN)_6]$) were obtained from Riedel Haen; troponin T (TnT), creatine kinase (CK-MB), cysteamine (Cys), bovine serum albumim (BSA), myoglobin (Myo) and *N*-Hydroxysuccinimide (NHS) from Fluka; *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDAC) from Alfa Aesar; and Creatine phosphate (Pcrea) and Creatine monohydrate (Crea) from Applichem.

2.3. Solutions

Stock solutions of 50 μg/mL CK-MB were prepared in PBS buffer pH 7.0. Electrochemical assays were performed with 5.0×10^{-3} mol/L $K_3[Fe(CN)_6]^{4-}$ and $K_4[Fe(CN)_6]^{3-}$ in PBS buffer. The selectivity study used 1.67 μg/mL CK-MB solutions prepared in buffer and solutions of possible interfering species, TnT (0.70 ng/mL), Myo (0.80 μg/mL) and BSA (0.30 μg/mL) prepared in the same buffer.

2.4. Design of biosensor Au-SPE

The working area of the SPE (gold) was cleaned by washing three times with ethanol. The gold surface of the WE was incubated in 25 mmol/L solutions of Cys for 2 h, at 25 °C, washed with deionized water several times and kept in 5×10^{-3} mol/L of activated Pcrea prepared in PBS buffer, pH 7.0, for 2.5 h, at room temperature. The carboxylic acid groups were activated by incubating for 90 min in an aqueous solution of 50 mmol/L EDAC and 25 mmol/L NHS at room temperature. The electrode was then rinsed thoroughly with distilled water to remove un-reacted species.

2.5. Binding approach

In order to evaluate the biological rebinding of the enzyme on the biosensor surface, the effective or apparent Michaelis–Menten constant (K_M) was determined by SWV from the (K_M). The Michaelis–Menten equation gave the parameters used to compare the biosensor performance (see Eq. (1)), where I_S is the current–density normalized, S is the biosensor response to a specified concentration of CK-MB and I_{max} is the maximum current–density response, obtained when all imprinted sites are saturated with the protein. The I_{max} reflected differences in the amount of active (not total) CK-MB bound in the sensor surface. K_M defined the concentration of substrate that gave half the I_{max} response.

$$I_S = \frac{I_{max}}{1 + K_M/[S]} \quad (1)$$

Changes in K_M are sensitive to variations in CK-MB access/binding, and have been interpreted in terms of barriers Pcrea/CK-MB (Sasso et al., 1990).

2.6. Electrochemical assays

CV and SWV measurements were conducted in 5.0 mmol/L of $[Fe(CN)_6]^{3-}$ and 5.0 mmol/L of $[Fe(CN)_6]^{4-}$, prepared in PBS buffer pH 7.4. For CV assays the potential was scanned from −0.7 to

+0.7 V, at 50 mV/s. In SWV studies, potentials were changed from 0.0 to +0.8 V, corresponding to a frequency of 50 Hz and step height of 150 mV. EIS assays were conducted with the same redox couple $[Fe(CN)_6]^{3-/4-}$ at a standard potential of 0.12 V, using a with amplitude 0.01 V RMS and a number of frequencies equal to 50, logarithmically distributed over a frequency range of 0.1–100 kHz. The impedance data were fitted to a Randles equivalent circuit using the implemented Nova software. All assays were conducted in triplicate.

2.7. Selectivity study

Selectivity studies were conducted by non-competitive assay. The interfering species tested were selected among those that may be found in biological fluids, such as TnT (0.7 ng/mL), Myo (0.8 μg/mL) and BSA (0.3 μg/mL). All assays were performed in triplicate.

2.8. Myo assay

The performance of the sensor was checked in synthetic serum samples, spiked with CK-MB in a range between 0.07 and 6.67 μg/mL. The serum was 1:10 diluted in PBS buffer and the measurements were performed by SWV measurements. All assays were performed in triplicate.

3. Results and discussion

3.1. Design of the sensory surface

The WE of the Au-SPE was modified by following the scheme in Fig. 1. It consisted of three different stages, starting with the formation of an amine layer, formed by incubating the gold surface in Cys. Its interaction with a gold substrate lead to the spontaneous formation of a closely packed monolayer via a strong gold–sulphur interaction between the –SH groups and gold.

The immobilization of the Pcrea on the modified surface was achieved by coupling its carboxylic acid group to a primary amine group at the modified gold electrode surface (Cys/Au-SPE). This was done via an amide linkage, formed after carboxylic acid activation by EDAC. This reaction forms a highly reactive *o*-acylisourea intermediate (Jiang et al., 2004) that reacts quickly with NHS to form a more stable ester. This ester undergoes nucleophilic substitution with any readily available amine group (on Cys), resulting in the formation of an amide bond between the modified Au-SPE and Pcrea.

Subsequently, the activated Pcrea solution was applied on the NH_2 functionalized Au electrode and left at room temperature for 2 h. The Pcrea/Cys/Au-SPE so-obtained was washed with PBS buffer to remove adsorbed Pcrea.

3.2. Control of the surface modification

Electrochemical biosensors provide an attractive resource to analyze the content of a biological sample due to the direct conversion of a biological event to an electronic signal, provided that these are setup properly and reproducibly. Over the past decades several sensing concepts have been developed (Grieshaber et al., 2008), by immobilizing organic films on a suitable support and following the electrical modifications occurring at the solid-state probe. Here, CV and EIS studies were made to follow the electrical behavior of the sensory surface.

CV voltammograms are shown in Fig. 2A. When compared to the redox probe in the bare gold, the consequent modification steps of the Au-SPE with Cys and Pcrea increased the peak-to-peak potential separation in the voltammograms, due the slow kinetics caused by the increased charge-transfer resistance. The cathodic and anodic peaks decreased with additional chemical modification.

EIS study acted as an indirect way of measuring the electrical alterations produced at each stage of chemical modification and Randle's equivalent circuit was adopted to model the

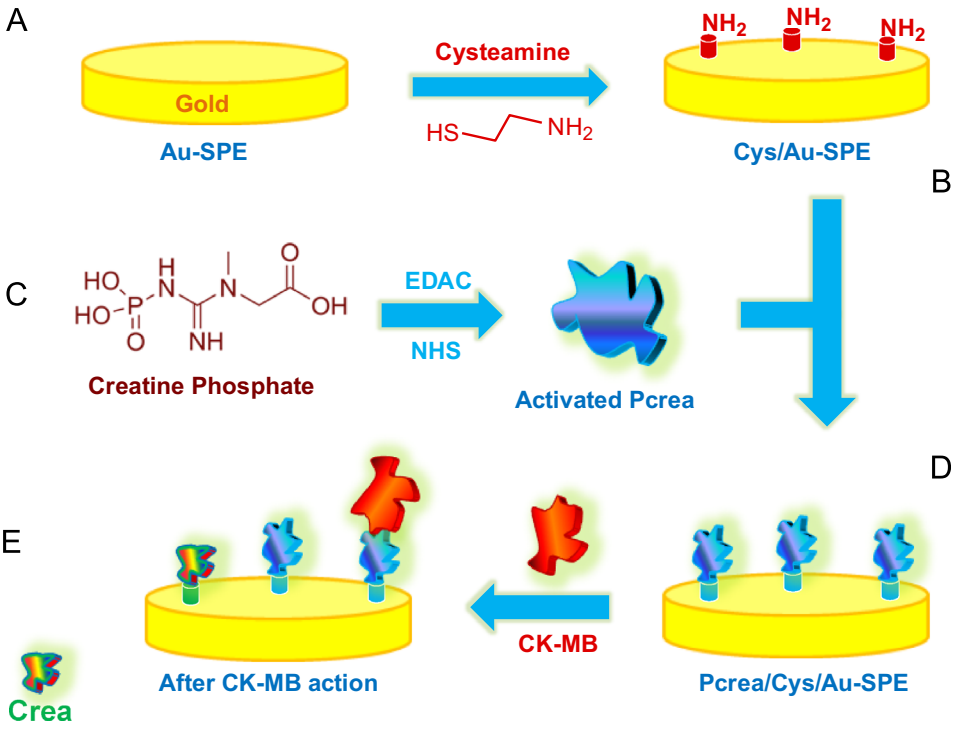

Fig. 1. Schematic illustration of the stepwise preparation of the biosensor: (A) bare gold of the Au-SPE; (B) amine monolayer formation; (C) activation of creatine phosphate; (D) creatine immobilization on the Cys/Au-SPE; and (E) reaction of CK-MB.

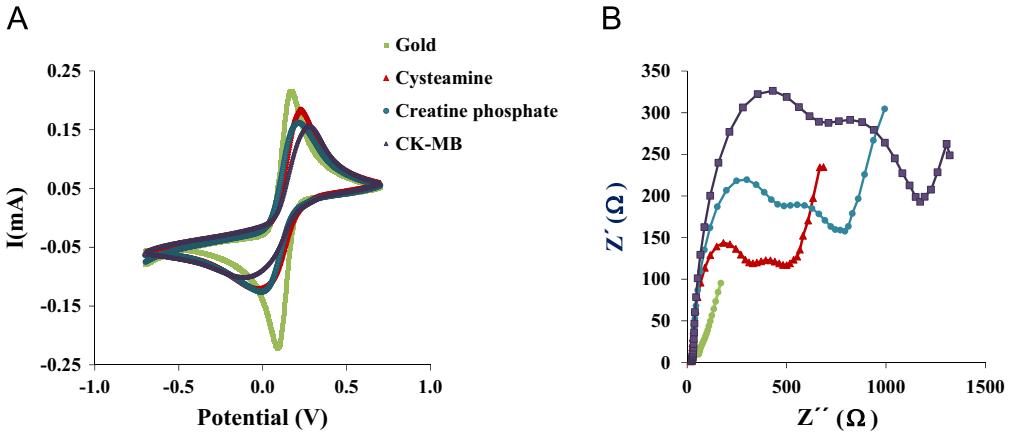

Fig. 2. Electrochemical CV voltammograms (A) and EIS (B) of the biosensor immobilization stages evaluated $[Fe(CN)_6]^{3-/4-}$, in PBS buffer pH 7.4.

physiochemical process occurring at the gold electrode surface because it is frequently used to interpret the electrochemical response of biosensing surfaces. EIS spectra were recorded for every step of the Au-SPE surface modification and the results presented as Nyquist plots (Fig. 2B). A large semicircle was observed at higher frequency range and indicated a charge-transfer controlled process (Panagopoulou et al., 2010), and this was followed by a smaller semi-circle at lower frequency range. The spectra also showed diffusion-controlled process from mass-transfer at the lowest frequencies under study (Suni, 2008). Overall, the bare gold electrode showed a very small semicircle area, suggesting a very fast electron-transfer process with a diffusional limiting step (Fig. 2B). Attachment of the Cys increased the electron transfer resistance, resulting in increase in the semicircular section of the Nyquist plot. The linkage of Pcrea produced an additional barrier for the redox probe access to the Au-SPE modified electrode. This resulted in an extra increase in the electron transfer resistance.

3.3. Creatine phosphate versus creatine

The main purpose of the present sensing system was to generate electrical changes deriving from a catalytic reaction between Crea and CK-MB, provided that Crea is immobilized at the sensing layer. Considering that CK-MB is a phosphotransferase, it would be reasonable to think that phosphate was required for the reaction to proceed. On the other hand, the same enzyme also catalyzes the reverse reaction, using as substrate Pcrea and producing Crea plus phosphate (that is readily transferred to any ADP present). So, instead of immobilizing Crea on the sensing gold layer and adding an external source of phosphate, Pcrea could be used. Furthermore, due to the presence of the phosphate group of Pcrea on modified SPE/Cys results in (i) oxidation/reduction peaks at lower potential, (ii) improvement of the electrocatalytic features and the (iii) increasing peak height of the biosensor. For comparison purposes, the electro-activity of the modified Crea/Cys/Au/SPE and Pcrea/Cys/Au/SPE were evaluated in PBS buffer pH 7.4 by SWV assay (Fig. 3). The modified Au-SPE with Pcrea showed an electrochemical peak with 0.0135 mA height, at 0.42 V. It was due to the presence of an electroactive phosphate compound, at that potential. The current peak obtained for Crea sensing layer was 0.00356 mA, at 0.46 V. The decrease in the peak height and potential shift compared to Pcrea are due to the absence of phosphate and the presence of a less-sensitive electroactive function. No peak was observed with the bare gold measurements.

Overall, the immobilization of Pcrea on Au/SPE was a simple and effective process enabling an easy and sensitive detection of the enzymatic reaction. In addition, and as long as CK-MB further

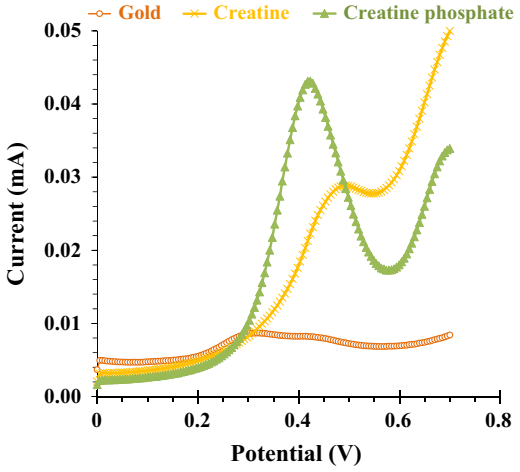

Fig. 3. SWV voltammogram in PBS buffer pH 7.4 for (i) bare gold SPE (o) (ii) creatine (×) and (iii) creatine phosphate (Δ).

reacts with this substrate, using Pcrea would avoid the need for a second electro-active species ($[Fe(CN)_6]^{3-/4-}$), responsible for generating the electro-analytical signal. This feature is also advantageous, once the mediators can cause passivation of the electrode surface, reducing its life time (Gomes-Filho et al., 2013).

3.4. Analytical performance of the sensory surface

In this work, the analytical performance of the sensor was evaluated with an electrochemical approach by following typical calibration curve procedures. The concentration range of CK-MB was within 0.19 and 28.8 μg/mL, and the equilibration time for the interaction between CK-MB and Pcrea/Cys/Au-SPE was set to 10 min.

SWV was selected among other voltammetric methods due its excellent sensitivity, rejection of background currents and speed. In addition, SWV provides rapid diagnostic information that can help identify the reversibility of an electrochemically active reaction (Babauta et al., 2012). The excitation signal in SWV consists of a symmetrical square-wave pulse of amplitude superimposed on a staircase waveform of step height, where the forward pulse of the square wave coincides with the staircase step. The net current was obtained with the difference between the forward and reverse currents and is centered on the redox potential. The peak height is directly proportional to the concentration of the electroactive species (Settle, 1997).

Overall, the presence of CK-MB decreased the peak current of Pcrea/Cys/Au-SPE, observed without CK-MB (Fig. 4). The peak

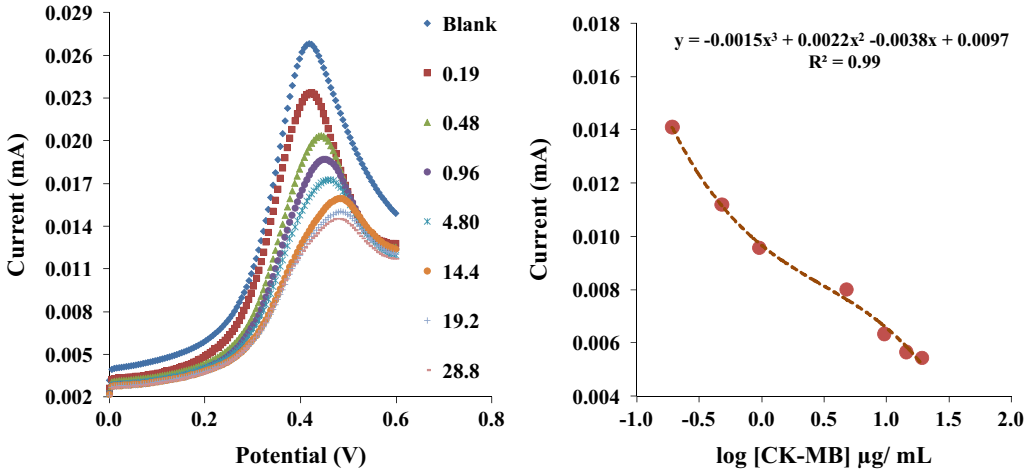

Fig. 4. Calibration curve of CK-MB biosensor obtained by SWV measurements in PBS buffer pH 7.0.

current reduction of the phosphate at the sensing surface was expected to be proportional to the concentration of CK-MB present in the sensing layer. This reduction could account the removal of phosphate from the surface by enzymatic reaction, thus reducing the amount of redox species present (Fig. 1E). On the other hand, the enzyme could also bind to the substrate and remain there, which would subsequently hinder the electrical current passage (Fig. 1E). Both of these would lead to a current decrease, justifying the observed response. In terms of overall analytical performance, it was possible to observe that this effect of CK-MB was consistent for all concentrations tested, ranging from 0.19 to 28.8 μg/mL. The sensor showed polynomial behavior from 0.48 to 28.8 μg/mL, with a slope -0.0035 ± 0.0099 μA × mL/μg and LOD 0.11 μg/mL.

Overall, this simple sensor, Pcrea/Cys/Au-SPE, showed high sensitive, in which the use of chemical mediators was not necessary for obtaining suitable electrochemical response.

3.5. Michaelis–Menten approach

The Michaelis–Menten behavior of the enzymatic system was checked out herein, but the enzyme concentration was increased instead of substrate due to the inverted configuration of the typical enzyme electrode. Increasing CK-MB concentrations shifted the current (in absolute value) of Pcrea/Cys/Au-SPE sensor to higher values, leading to a saturation curve that followed the Michaelis–Menten hyperbolic behavior and K_M is the Michaelis–Menten constant and it corresponded to the concentration of substrate when the reaction velocity was equal to one half of the maximal velocity observed. The value of this constant allowed to measuring how well the CK-MB complexed with Pcrea and the inherent affinity. A low K_M indicated a large binding affinity, as the reaction approached V_{max} more rapidly, while a high K_M indicated that the biosensor material did not bind as efficiently with the CK-MB, and I_{max} would only be reached if the substrate concentration CK-MB would be high enough to saturate the material. The results were compared with the Crea/Cys/Au-SPE biosensor. The K_M and I_{max} values for Pcrea/Cys/Au-SPE biosensor were 0.9 μA × mL/μg) and 0.11 μA and 4.0 μA × mL/μg) and 0.03 μA for Crea/Cys/Au-SPE respectively using Michaelis–Menten equation. The results demonstrate that the biosensor and CK-MB have higher affinity for Pcrea than Crea biosensor (Fig. 5).

3.6. Selectivity study

The selectivity of the sensor was evaluated in a non-competitive environment. The interfering species tested were selected among

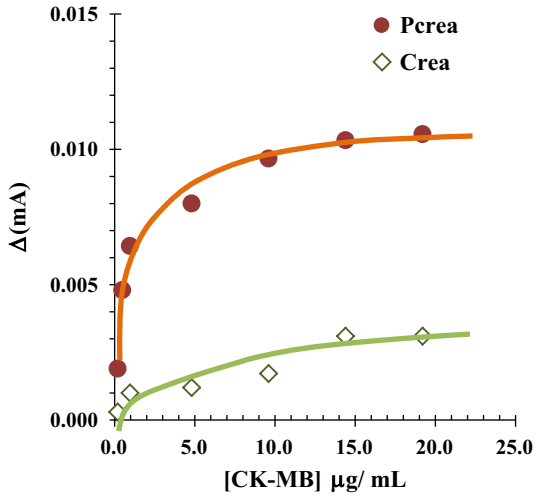

Fig. 5. Typical Michaelis–Menten profile of the sensory surface for increasing enzyme concentration.

those that may be found in biological fluids, such as TnT, BSA, and Myo. The non-competitive assay was used with CK-MB (1.67 μg/mL), TnT (0.7 ng/mL), BSA (0.3 μg/mL) and Myo (0.8 μg/mL). This interference study uses concentration levels of cardiac biomarkers that may exist in biological fluids in ischemic heart episodes.

The time given for CK-MB or interfering species to interact was set to 10 min. After this, the peak current changed by +9.8% for BSA assays, −3.8% for TnT and +4.0% for Myo. The measurements were performed in triplicate using different gold SPE, to ensure good reproducibility (see Fig. 6).

3.7. CK-MB assay

The applicability of the sensor was tested by determining CK-MB in synthetic urine. For this purpose, calibration curves were recorded in standard solutions prepared in synthetic urine instead of buffer. The obtained calibration showed good analytical features, producing LLLR, LOD and slope values of 0.05 μg/mL, 0.48 μg/mL and −0.001 μA/decade [CK-MB, μg/mL], respectively. The slope was smaller than the calibration in buffer, but this is in agreement with the effect of high amounts of BSA on the sensor performance.

Synthetic serum samples spiked with different concentrations of CK-MB ranging between 0.07 and 6.67 μg/mL were analyzed by

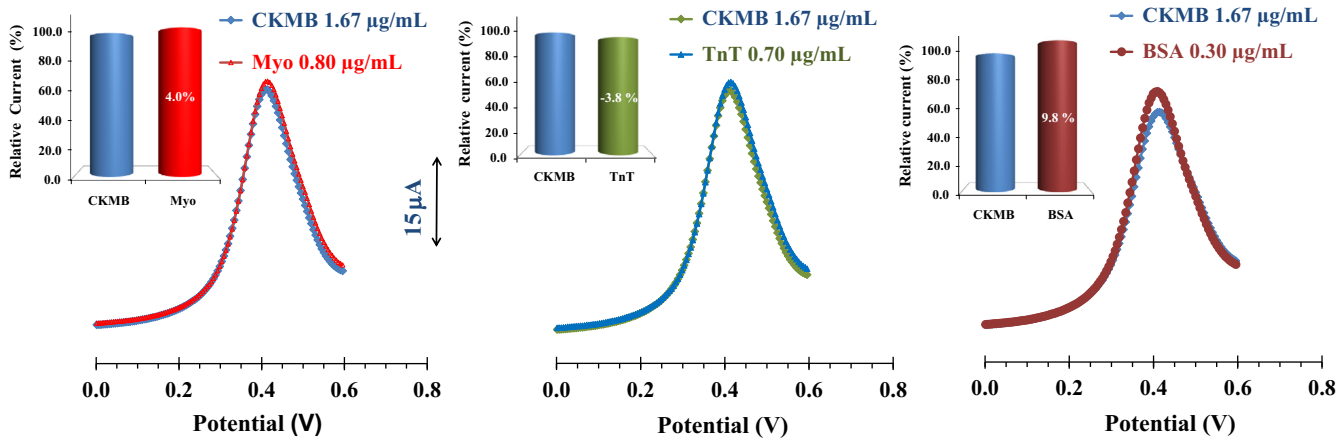

Fig. 6. Selectivity data for Myo, TnT and BSA.

SWV. Overall, the results obtained suggested that the sensor could offer useful readings under real conditions.

4. Conclusions

A simple method fast for screening CK-MB in POC with electrochemical transduction was developed. The CK-MB determination has been achieved in mediator free-conditions due the redox proprieties of the Pcrea. Good performance in terms of selectivity and sensitivity were observed.

The biosensor was successfully applied to biological fluids, showing good stability at room temperature and excellent sensitivity and selectivity. This work opened new horizons at the production of cost-effective biosensors for cardiac biomarker detection in POC due its simplicity, robustness and feasibility and for other biosensors aiming at quantifying proteins with catalytic activity.

Acknowledgments

One of the authors (FTCM) gratefully acknowledges Fundação para a Ciência e Tecnologia for the financial support (Ph.D. grant reference SFRH/BD/66735/2009).

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