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Molecular imprinting coupled with electrochemical analysis for plasma samples classification in acute myocardial infarction diagnostic

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ABSTRACT

Electroanalysis of myoglobin (Mb) in 10 plasma samples of healthy donors (HDs) and 14 plasma samples of patients with acute myocardial infarction (AMI) was carried out with screen-printed electrodes modified first with multi-walled carbon nanotubes (MWCNT) and then with a molecularly imprinted polymer film (MIP), viz., myoglobin-imprinted electropolymerized poly(*o*-phenylenediamine). The differential pulse voltammetry (DPV) parameters, such as a maximum amplitude of reduction peak current (**A**, nA), a reduction peak area (**S**, nA × V), and a peak potential (**P**, V), were measured for the MWCNT/MIP-sensors after their incubation with non-diluted plasma. The relevance of the multi-parameter electrochemical data for accurate discrimination between HDs and patients with AMI was assessed on the basis of electrochemical threshold values (this requires the reference standard method (RAMP® immunoassay)) or alternatively on the basis of the computational cluster assay (this does not require any reference standard method). The multi-parameter electrochemical analysis of biosamples combined with computational cluster assay was found to provide better accuracy in classification of plasma samples to the groups of HDs or AMI patients.

1. Introduction

Cardiovascular diseases take the first place among causes of death of the population in Russia (www.gks.ru) and cause nearly a half of the total number of death in Europe (www.ehnheart.org). In the last decade, considerable efforts have been aimed at identification of cardiac markers to be used for diagnostics at cardiac pains, thus contributing to more exact definition of risk criteria. The cardiac marker is a biological substance whose level in blood is considerably increased during a cardiovascular disease or right after a damage of the cardiac muscle (McDonnell et al., 2009). Up to now, 177 different substances – biomarkers of cardiovascular diseases are known (Aldous, 2013).

Myoglobin (Mb) is a heme-containing redox protein with molecular weight of 17.8 kD. Mb is present in serum of healthy individuals in rather low concentrations (from 70 to 90 ng/ml (4–5 nM) to 200 ng/ml (11 nM)). The most researchers adhere to an average value of the Mb concentration of about 100 ng/ml (6 nM) (Matveeva et al., 2005). Meanwhile, the Mb concentration in blood can increase by a factor of 4–10 from a baseline value 1–3 h after the appearance of initial symptoms of the acute myocardial infarction (AMI) and reaches the maximum value in the period between 6 and 12 h. This property of Mb allows it to be considered as one of the earliest markers of AMI, which can be used as an early confirmation of the AMI onset (Aldous, 2013). Hence, the level of Mb is a very promising protein marker indicating severity of a heart attack at an early stage of its development, which is important for choosing optimal treatment of AMI patients.

To cover the whole range of the Mb concentrations, the affine partner (antibody) having low binding constants is necessary to be taken for adequate target recognition. As shown earlier, Mb was quantified at the biological level in undiluted plasma using electrochemical immunosensors with immobilized anti-Mb (antibodies) (Shumyantseva et al., 2010, 2015b; Suprun et al., 2010). In spite of many advantages of immunoassays, the quality of the analysis is very dependent on the antibody nature, their purity, producing company, etc. One should also mention that immunoassay is rather expensive as well as time- and labor-consuming.

Molecularly imprinted polymers (MIPs) are extensively used as synthetic polymer-based receptors or artificial antibodies (Erdossy et al., 2016; Ma et al., 2016; Turner et al., 2006; Uzun and Turner,

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2016). They are typically prepared via polymerization of suitable monomers in the presence of template molecules, followed by extraction of the template. Each step of this rather cheap procedure can be standardized to give reproducible binding and analytical response. MIP-techniques, being a promising alternative to classical immunoanalysis, are applied for cardiac biomarkers detection (Karimian et al., 2014, 2013; Shumyantseva et al., 2016a, b, 2015a). A combination of the MIP-techniques with well-established electrochemistry of hemoproteins (Han et al., 2002; Liu et al., 2004; Nassar et al., 1995; Rusling and Nassar, 1993; Shen et al., 2002; Suprun et al., 2014; Wu et al., 2006; Zhao et al., 2003) is also intensively developed and published for proteins other than Mb, e.g., cytochrome *c* and hemoglobin (Bosserdt et al., 2013; Reddy et al., 2011).

Earlier, we have developed a method for recognition and quantitative detection of skeletal/cardiac Mb by means of MIP-sensors composed of the electropolymerized *o*-phenylenediamine (*o*-PD) as a Mb biorecognition element (Shumyantseva et al., 2016a, b, 2015a). The detailed study of electropolymerization of *o*-PD/Mb by the combined electrochemical quartz crystal microbalance with monitoring dissipation (EQCM-D) technique allows us to come to a well-defined and easily controlled electrochemical fabrication of MIP on the surface of a screen-printed electrode (SPE). Similar MIP-sensors conjugated with multi-walled carbon nanotubes (MWCNT) were further developed to improve the sensitivity of Mb detection. Due to unique properties of MWCNT, such as superior conductivity, facilitated electron transfer, and high surface area (Lawal, 2016), the sensitivity of the designed sensor systems was considerably improved from 2.0×10^{-4} A/nmol for the MIP-sensor (Shumyantseva et al., 2016b, 2015a) to 1.5×10^{-2} A/nmol for the MWCNT/MIP-sensor (Shumyantseva et al., 2016a), the analytical responses being measured by high sensitive differential pulse voltammetry (DPV). Mb binding to the MWCNT/MIP-sensor was described by a hyperbolic curve having two linear regions in the ranges of 1×10^{-11}–5×10^{-10} M and 5×10^{-10}–5×10^{-8} M of Mb (Shumyantseva et al., 2016a). These ranges cover the whole window of the Mb concentrations in plasma samples of HDs and AMI patients. Thus, our MWCNT/MIP-sensor system can serve as a robust and versatile sensor element and can be applied for analysis of biological samples.

New types of “point-of-care” or ‘detect-to-protect” biosensors are currently intensively developed. A signal (affine binding, catalysis, inhibition, etc.) they measure is processed and referred to a certain group by the “yes/no” principle. Among others, clustering techniques are widely used in biological studies, from discovering molecular subtypes of cancer (Verhaak et al., 2010) to elucidating proteins associated with toxic effects of drugs (Ivanov et al., 2016).

The traditionally used immunoassay gives a univariate (one-dimensional) numerical signal, like absorbance, fluorescence, staining, etc., while a multi-dimensional numerical signal can be obtained by electrochemistry. Along with other electrochemical techniques, DPV provides a number of analytical parameters, viz., the maximum amplitude of cathodic current reduction, the area of the peak corresponding to reduction of a target molecule (hemoprotein; Mb in our case), and the peak potential. This key distinction from other analytical techniques, like immunoassay, seems to be beneficial for increasing a number of data to be analyzed for the precise quantification and the further classification of biosamples.

In this paper, we describe the analysis and the following classification of 10 plasma samples of HDs and 14 plasma samples of patients with AMI. Electrochemical DPV parameters, the maximum amplitude of cathodic current, the peak area corresponding to reduction of hemoprotein and the peak potential, measured by the MWCNT/MIP-sensor were analyzed. Within the scope of this article, clustering was used to assess relevance of multi-parameter electrochemical data for an accurate discrimination between the HDs and the patients with AMI (see Scheme 1).

2. Materials and methods

2.1. Materials

Mb from equine skeletal muscle and *o*-PD were obtained from Sigma-Aldrich Co. MWCNT with the external diameter of 10–15 nm, the internal diameter of 2–6 nm, and the length of 0.1–10 μm were purchased from Arkema Inc. Sigma-Aldrich. All other chemicals were of analytical grade and used without further purification. All aqueous solutions were prepared using Milli-Q water (18.2 MΩ cm) purified with a Milli-Q water purification system by Millipore.

Plasma samples of HDs were obtained from blood bank while plasma samples of patients with AMI were obtained from a hospital from the patients with the confirmed AMI diagnosis. The informed signed consent was received from each of the patients. Plasma of the HDs and the patients with AMI was collected after centrifugation of blood with ethylenediaminetetraacetic acid as anticoagulant for 10 min at 3000 rpm. The concentrations of Mb in plasma samples were determined with the bench-top lateral flow RAMP® (Response Biomedical Corp) immunoassay according to the guideline.

2.2. Preparation of MWCNT/MIP-sensors

MWCNT/MIP-sensors were prepared via electropolymerization of *o*-PD in the presence of Mb as a template on the surface of the SPE/MWCNT, followed by extraction of the template with an alkali-ethanol solution. The detailed procedure of MWCNT/MIP-sensors fabrication is described in the SI. For further details, see also references (Shumyantseva et al., 2016a, b, 2015a).

2.3. Electrochemical measurements

For the electrochemical analysis of Mb, unfrozen samples of undiluted plasma of the HDs or the patients with AMI were dropped onto the MWCNT/MIP-sensor surface (2 μl) and the sensors were incubated for 15 min at + 37 °C. The electrochemical analysis of the bound Mb was performed after the incubation of the MWCNT/MIP-sensors for 15 min in 100 mM phosphate buffer saline (PBS) of pH 7.4 via direct electrochemical detection of DPV peak corresponding to the reduction of Fe^{3+} of hemoprotein in the range of potentials from −0.4 V to −0.6 V. Electrochemical measurements were performed with a potentiostat/galvanostat AUTOLAB12 (Metrohm Autolab, The Netherlands) equipped with the software GPES (version 4.9.7). The MWCNT/MIP-were tested in the DPV regime using pulse amplitude of 25 mV, potential step of 1 mV, and the pulse duration of 50 ms. All potentials were referred to the Ag/AgCl reference SPE. The numerical electrochemical data were based on at least three independent electrochemical experiments.

2.4. Clustering assay

The values of the cathodic peak area, the maximum amplitude of reductive current, and/or the cathodic peak potential were used as descriptive features for samples obtained from the HDs and the patients with AMI. Prior to clustering itself, the data were normalized by subtracting the mean and dividing by the standard deviation (SD) to provide the significance for both features with different orders of magnitude. Spherical *k*-Means Clustering was used to distribute records from the dataset into clusters. The Spherical *k*-Means Clustering was a part of the package “skmeans” for R-language, which previously had been described by its authors in great details (Hornik et al., 2012). Silhouette widths were used as a measure of the consistency of the data within clusters to select the appropriate number of clusters (Rousseeuw, 1987). A more detailed description of Spherical *k*-Means Clustering algorithm is given in the SI.