ISSN 1607-6729, Doklady Biochemistry and Biophysics, 2016, Vol. 468, pp. 213–216. © Pleiades Publishing, Ltd., 2016.
Original Russian Text © V.V. Shumyantseva, T.V. Bulko, L.V. Sigolaeva, A.V. Kuzikov, A.I. Archakov, 2016, published in Doklady Akademii Nauk, 2016, Vol. 468, No. 4, pp. 466–469.

BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

# Electroanalysis of Myoglobin Based on Electropolymerized Molecularly Imprinted Polymer Poly-*o*-Phenylenediamine and Carbon Nanotubes/Screen Printed Electrode[1]

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Received December 15, 2015

**Abstract**—Electroanalysis of myoglobin as a marker of acute myocardial infarction by means of screen-printed electrodes modified with multiwalled carbon nanotubes and polymeric artificial antibodies is developed. Plastic antibodies to myoglobin (molecularly imprinted polymers, MIPs) based on *o*-phenylenediamine were produced by electropolymerization. Molecular imprinting technology in biosensor analysis was used as alternative to natural receptors (namely, antibodies) and demonstrated high sensitivity ($1.5 \times 10^{-2}$ A/nmol of myoglobin) and selectivity.

**DOI:** 10.1134/S1607672916030157

The cardiovascular diseases (CDs) are among the main problems of the Russian and international health care. CDs and acute myocardial infarction (AMI) pose real threat to health and reduce the life expectancy of the person. According to data of the Federal State Statistics Service (www.gks.ru), cardiovascular diseases rank first among the causes of death of the population in Russia and Western Europe (www.ehnheart.org). In this regard, the accurate evaluation of patients with AMI symptoms is of great clinical importance.

Myoglobin is a tissue-specific low-molecular-weight hemoprotein (17.8 kDa), one of the cardiac marker proteins [1, 2]. The myoglobin concentration in blood increases as early as 1–3 h upon symptom onset [3]. The increase in the myoglobin concentration is the criterion of the AMI sharp phase. Myoglobin is present at very low concentrations (from 70–90 ng/mL, 4–5 nM) to 200 ng/mL, 11 nM) in serum of healthy individuals. However, the majority of researchers adhere to an average value of about 100 ng/mL (6 nM) [4, 5]. Myoglobin concentration in blood can increase 4–10 times from the baseline value during AMI. Myoglobin level is a very promising protein marker for the determination of extensiveness of a heart attack at an early stage of its development, which is important for the selection of optimum treatment of patients [6].

Various approaches are used for Mb registration, such as mass spectrometry, liquid chromatography, colorimetry, surface plasmon resonance, and electroanalysis [7]. The affinity capture agents, as a rule, are the myoglobin-specific (anti-myoglobin) antibodies. Molecular imprinting technology in the biosensor analysis is an alternative to the natural affine reagents as recognition elements [8]. Earlier, we applied the molecularly imprinted polymers (MIPs) based on electropolymerized *o*-phenylenediamine as biorecognition reagent for the analysis of myoglobin [9]. MIPs conjugated with carbon nanotubes (CNTs) were developed for improvement of sensitivity for analysis of the cardiac marker myoglobin. CNTs possess a number of remarkable properties, such as high electrical conductivity and ability to transfer electron, and extraordinarily large specific surface area (the specific surface area of CNTs is >600 $m^2/g$, while the specific surface area of absorbent carbon is 20–50 $m^2/g$), high mechanical properties, chemical inertness, thermal stability, and biocompatibility [10, 11].

Electrochemical measurements were performed with an AUTOLAB12 potentiostat/galvanostat (Metrohm Autolab, The Netherlands) equipped with the GPES software (version 4.9.7). Three-contact screen-printed electrodes (ColorElectronics Research and Production Enterprise, LLC, Russia, http://www.colorel.ru) with graphite working and auxiliary electrodes and reference Ag/AgCl electrode were used.

[1] The article was translated by the authors.

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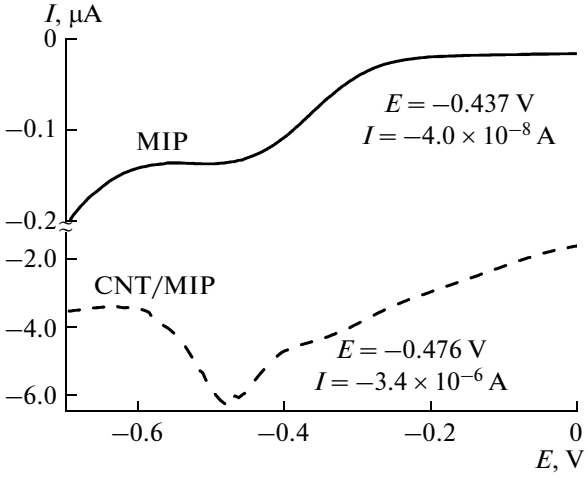


**Fig. 1.** SWV curves (the reduction peak) of the electrodes modified with MIP (—) and CNT/MIP (--) after their incubation with myoglobin ($1 \times 10^{-7}$ M, 15 min, 37°C)) and the washing stage for the elimination of non-specific protein binding. The potentials was ranged from −0.7 to +0.1 V (vs. Ag/AgCl), the scan rate was 50 mV/s, the frequency was 10 Hz, the amplitude was 20 mV, and the step potential was 5 mV.

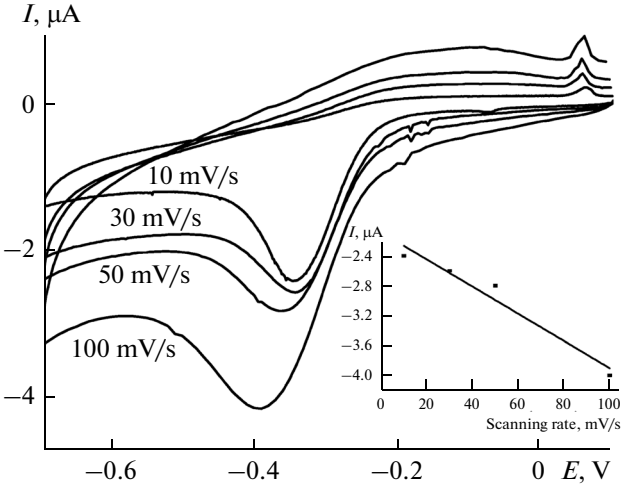


**Fig. 2.** CV of CNT/MIP electrodes after binding with $10^{-8}$ M myoglobin, 15 min, 37°C, at scan rates of 10, 30, 50, and 100 mV/s. Inset: relationship between the scan rate and the cathodic peak current of CNT/MIP electrode in 0.1 M potassium phosphate buffer, 0.05 M KCl.

All measurements were performed in 0.1 M potassium phosphate buffer containing 0.05 M NaCl (pH 7.4). CNTs with an external diameter of 10–15 nm, internal diameter of 2–6 nm, and length of 0.1–10 μm were used (Arkema Inc. SIGMA-ALDRICH). Since CNTs are insoluble in water and organic solvents, to obtain a homogeneous coating, we prepared CNT suspension in chloroform or dimethylformamide (1 mg/mL) by means of ultrasonic disintegration. Then, 2 μL of CNT suspension in organic solvent was applied on the graphite working electrode surface, and electropolymerization of *o*-phenylenediamine with myoglobin (MIP) or without myoglobin (NIP) was carried out. Electrosynthesis of films was carried out in 0.5 M acetate buffer (pH 5.2) as described earlier [9]. The electrodes without molecular fingerprints (NIPs) were prepared in a similar way using the corresponding volume of 0.5 M acetate buffer (pH 5.2) instead of the myoglobin solution.

Analysis of myoglobin was carried out through a direct electrochemical detection of the peak corresponding to the reduction of $Fe^{3+}$ of hemoprotein in the range of potentials from −0.4 to −0.6 V by cyclic voltammetry (CV) and square wave voltammetry (SWV). The electrodes modified by CNT/MIP show higher value of the SWV maximum current in comparison with the electrodes without CNT ($3.4 \times 10^{-6}$ and $4.0 \times 10^{-8}$, respectively) after binding with $10^{-8}$ M myoglobin (Fig. 1). Myoglobin reduction potentials of MIP and CNT/MIP modified electrodes were close: $E = -0.44 \pm 0.08$ V, and $E = -0.48 \pm 0.06$ V, respectively.

The analysis in the presence of oxygen allows registering electrocatalytic properties of myoglobin as metalloprotein, which leads to the increase in the sensor signal [12, 13]. The sensitivity of MIP and CNT/MIP electrodes corresponds to $2 \times 10^{-4}$ A/nmol and $1.5 \times 10^{-2}$ A/nmol, respectively.

The imprinting factor, evaluated as the ratio of the maximum amplitudes of the SWV currents and corrected for the baseline, $I_{max(MIP)}/I_{max(NIP)}$, was found to be 2–4.

Figure 2 shows the CV of CNT/MIP electrodes at different scan rates. Reductive peak responses of CV were attributed to the redox process of the electroactive center of myoglobin bound with poly-*o*-phenylenediamine imprinted films. The cathodic peak currents were linearly proportional to the scan rate changed from 10 to 100 mV/s (Fig. 2, inset). This result indicates that the electrochemical behavior of myoglobin in CNT/MIP films is a surface-controlled process attributed to protein film voltammetry [14]. The cathodic peak potential of myoglobin shifted to the negative direction with the increase of the scan rate, indicating that the reductive process is irreversible in accordance with the scheme [15].

$$[\text{myoglobin}-\text{Fe(III)}] + 1e \rightarrow [\text{myoglobin}-\text{Fe(II)} + O_2].$$

The quantitative analysis of myoglobin binding with MIPs was carried out after the incubation of electrodes in myoglobin solution in the concentration range of $1 \times 10^{-11}$–$1 \times 10^{-6}$ M. To remove the nonspecific myoglobin sorption, the sensors were washed with electrolyte buffer and then with water, after which they were examined by SWV. The binding kinetics of myoglobin is presented by the saturation curve fitted by the Langmuir isotherm ($R^2 = 0.94$) (Fig. 3).

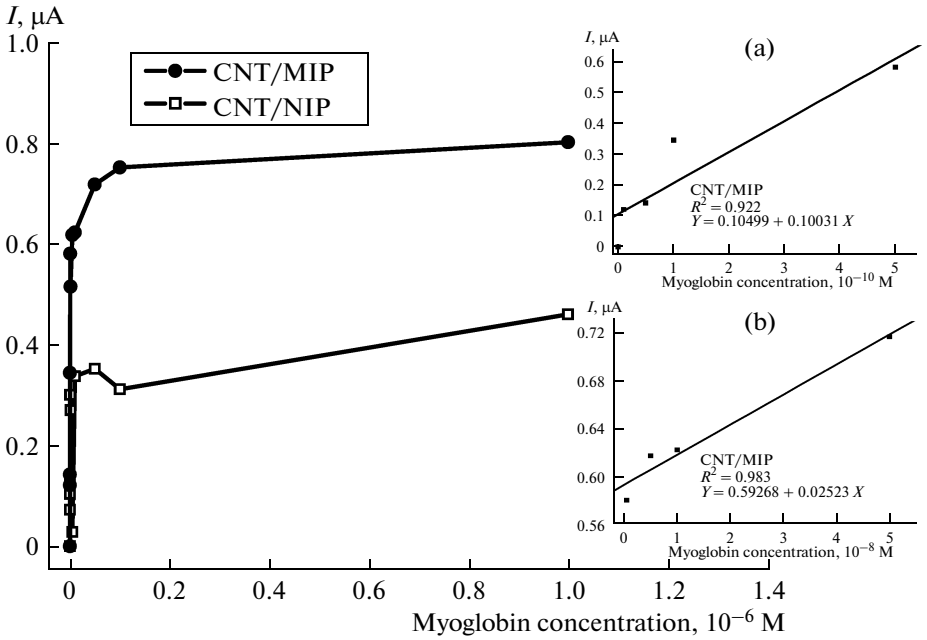


**Fig. 3.** The dependence of the maximum SWV current (the reduction peak) of the CNT/MIP (–●–) and CNT/NIP (–□–) electrodes on the myoglobin concentration in the range of $1 \times 10^{-11}$–$1 \times 10^{-6}$ M. Inset: (a) linear relationship between the maximum SWV current and the myoglobin concentration in the range of $1 \times 10^{-11}$–$5 \times 10^{-10}$ M; (b) linear relationship between the maximum SWV current and the myoglobin concentration in the range of $5 \times 10^{-10}$–$5 \times 10^{-8}$ M.

The concentration dependence has two linear ranges: of $1 \times 10^{-11}$–$5 \times 10^{-10}$ ($R^2 = 0.922$ (Fig. 3, inset a)) and $5 \times 10^{-10}$–$5 \times 10^{-8}$ ($R^2 = 0.983$ (Fig. 3, inset b)). The binding of myoglobin with the CNT/NIP electrode is poorly fitted by the Langmuir isotherm ($R^2 = 0.86$).

The equilibrium dissociation constant ($K_d$,CNT/MIP) for the interaction of myoglobin with the CNT/MIP electrodes was calculated as the myoglobin concentration at which the SWV current $I_{1/2}$ corresponds to a half of the maximum value of $I_{max}$ and was found to be $(9.8 \pm 2.6) \times 10^{-11}$ M. As we have shown earlier, $K_d$/MIP for MIP electrodes was found to be $(2.4 \pm 0.5) \times 10^{-8}$ M [9].

Detection of cardiac markers is usually carried out in plasma or serum. Albumin is present in plasma at concentrations of 40–50 mg/mL and it is one of interfering proteins in myoglobin binding with MIP. The analysis of myoglobin in the presence of human serum albumin, HSA ($10^{-7}$ M Mb + $10^{-7}$ M HSA) was carried out. Figure 4 shows that HSA reduces the signal strength of the recovery SWV peak of myoglobin showing the properties of an "insulator" and a modifier of the surface of CNT/MIP electrode. However, after the incubation of CNT/MIP electrodes in the electrolyte buffer for removal of albumin, the reductive current of myoglobin increases and reaches the maximum value for 60 min. These results can be interpreted as an evidence of a more effective interaction of myoglobin (MW 17.8 kDa) with MIP films and the removal of loosely bound interfering HSA with a high molecular weight (MW 65 kDa) from the surface of the myoglobin-imprinted film. The results obtained need to be when developing the protocol for the detection of myoglobin in plasma or serum of patients with AMI.

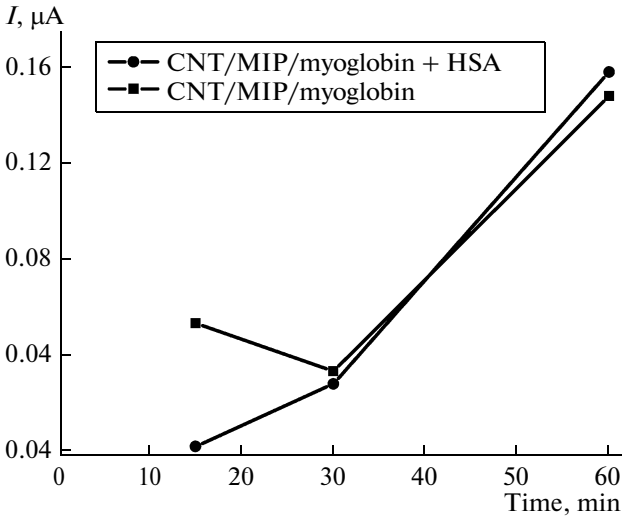


**Fig. 4.** Effect of albumin removal on the peak current intensity of myoglobin testing. CNT/MIP electrode with immobilized $1 \times 10^{-7}$ M myoglobin + $1 \times 10^{-7}$ M HSA, 2 μL (–●–) and CNT/MIP electrode with immobilized $1 \times 10^{-7}$ M myoglobin, 2 μL (–■–). HSA was removed by washing for 60 min.

In summary, the conjugation of MIP with CNT-modified electrodes exhibited essential increasing in the myoglobin sensing by means of electroanalysis. The CNT/MIP performance is attributed to the effective orientation of myoglobin in poly-*o*-phenylenediamine-imprinted films and the efficient conductivity of CNT-modified electrodes. CNT/MIP films can be considered as an alternative to natural antibodies in biosensor analysis.

## ACKNOWLEDGMENTS

This work was supported by the Ministry of Education and Science of the Russian Federation in the framework of the project no. 14.576.21.0045 (the unique identifier RFMEF1576I4X0045).

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