Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2020, **30**, 299–301

Mendeleev Communications

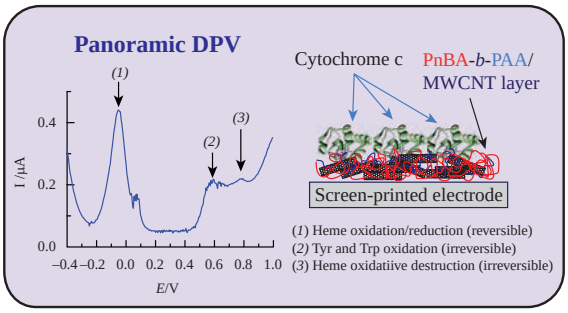
Electrochemical fingerprint of cytochrome *c* on a polymer/MWCNT nanocomposite electrode

Victoria V. Shumyantseva,*[a,b,c] Tatiana V. Bulko,[a,c] Alexey V. Kuzikov,[a,b,c] Rami A. Masamrekh,[a,b,c] Dmitry V. Pergushov,[c] Felix H. Schacher[d,e,f] and Larisa V. Sigolaeva[a,c]

[a] *Institute of Biomedical Chemistry, 119121 Moscow, Russian Federation. Fax: +7 499 245 0857; e-mail: viktoria.shumyantseva@ibmc.msk.ru*
[b] *N. I. Pirogov Russian National Research Medical University, 117997 Moscow, Russian Federation*
[c] *Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation*
[d] *Institute of Organic Chemistry and Macromolecular Chemistry, Friedrich-Schiller-University Jena, D-07743 Jena, Germany*
[e] *Jena Center for Soft Matter, Friedrich-Schiller-University Jena, D-07743 Jena, Germany*
[f] *Center for Energy and Environmental Chemistry, Friedrich-Schiller-University Jena, D-07743 Jena, Germany*

DOI: 10.1016/j.mencom.2020.05.012

The electroactivity of cytochrome *c* on screen-printed graphite electrodes modified with stable dispersions of multi-walled carbon nanotubes in aqueous solution of amphiphilic poly(*n*-butyl acrylate)$_{100}$-*block*-poly(acrylic acid)$_{140}$ diblock copolymers was estimated. Using a broad potential window, the reduction and oxidation of heme iron at a near-zero potential, the electrooxidation of Tyr and Trp at +0.6 V and the electrooxidation of heme at +0.8 V were detected in a wide concentration range of 1–100 μM with a limit of detection of 1–2 μM. Such a multipoint detection can be used as an electrochemical fingerprint of cytochrome *c* for its electrochemical recognition and quantification in complex (bio)-chemical analytes.



Keywords: cytochrome *c*, modified electrode, multi-walled carbon nanotubes, amphiphilic diblock copolymer, label-free detection, screen-printed electrodes, electrooxidation, electron transfer.

Cytochrome *c*, a metalloprotein with a covalently attached heme prostetic group, plays a key role as an electron carrier and a pro-apoptotic protein in cell fate.[1,2] Due to its antioxidative properties, cytochrome *c* belongs to the defense group of enzymes in mitochondria, and this heme protein is a marker of myocardial infarction, cardiac arrest and resuscitation, mitochondria injury, organ damage, and apoptosis of different generation, including the influence of anticancer drugs.[2–4] Cytochrome *c* biosensors are promising drug-screening tools for the analysis of new anticancer drug candidates. With these biosensors one can monitor cytochrome *c* release from mitochondria in lysates of cells and carry out general mechanistic studies of apoptotic processes.[5–7] However, the heme of cytochrome *c* is buried in a protein polypeptide chain; hence, electron transfer in this metalloprotein to any electrode is diminished or blocked.[8] This renders direct electron exchange between the heme and the electrode challenging, which would be much desired as the simplest reagent-less detection of cytochrome *c* (direct electron-transfer biosensor). Hence, new nanomaterials and electrode modification techniques for the highly sensitive detection of cytochrome *c* are of great demand.

Within the mitochondrial membrane, cytochrome *c* having a high isoelectric point of about 10 is associated with anionic phospholipids, such as phosphatidylserine, cardiolipin, or phosphatidic acid.[4,9] Here, we report the electroanalysis of cytochrome *c* based on the modification of a screen-printed graphite electrode with a stable dispersion of multi-walled carbon nanotubes (MWCNTs) in aqueous solutions of anionic amphiphilic poly(*n*-butylacrylate)$_{100}$-*block*-poly(acrylic acid)$_{140}$ (PnBA$_{100}$-*b*-PAA$_{140}$) diblock copolymer micelles. We assumed that the amphiphilic diblock copolymer[10] facilitates the dispersion of MWCNTs due to (i) hydrophobic interactions of the poly(*n*-butyl acrylate) block with the MWCNTs surface and (ii) mimicking of the natural microenvironment of cytochrome *c* similar to anionic phospholipids *via* ionic interactions with poly(acrylic acid) (PAA) segments [Figure 1(*a*)]. We reported earlier a

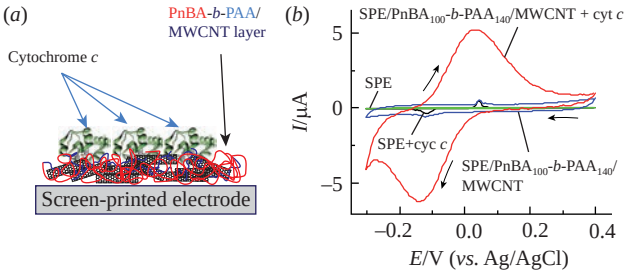


Figure 1 (*a*) PnBA$_{100}$-*b*-PAA$_{140}$/MWCNT nanocomposite electrode with electrostatically bound cytochrome *c*; (*b*) cyclic voltammograms of heme iron reduction/oxidation for 2 mM cytochrome *c* solution deposited on SPE/PnBA$_{100}$-*b*-PAA$_{140}$/MWCNT. Scan rate, 50 mV s^{-1}.

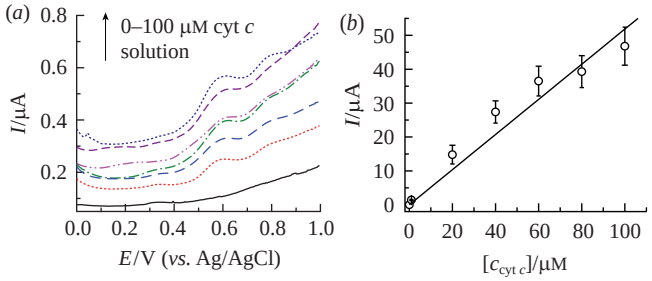


Figure 2 (*a*) DPV responses of 0–100 μM cytochrome *c* solution on SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT; (*b*) anodic DPV peak current plotted *vs.* cytochrome *c* concentration in a range of 0–100 μM (R^2 = 0.96).

similar approach to electrode surface modification with amphiphilic ionic diblock copolymers and demonstrated their potential for the analysis of dsDNA[11] and myoglobin.[12] We expected that the resultant construct would enhance the electrochemical reactions of cytochrome *c* on the electrode surface and improve the electroanalysis of this protein.

Three-pronged screen-printed electrodes (SPEs) from Color Electronics (Russia, http://www.colorel.ru) were used for the electrode preparation. $PnBA_{100}$-*b*-PAA_{140}/MWCNT composite preparation and SPE modification were carried out according to published procedures.[11]

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were carried out using an Autolab PGSTAT 12 potentiostat/galvanostat (Metrohm Autolab, the Netherlands) with the GPES software (version 4.9.7). The electrochemical measurements were performed at room temperature in a 0.1 M potassium phosphate buffer (PBS) with 50 mM NaCl as a supporting electrolyte. All potentials are given *vs.* a reference electrode (Ag/AgCl).

Cytochrome *c* immobilized on $PnBA_{100}$-*b*-PAA_{140}/MWCNT nanocomposite demonstrated direct electron transfer with symmetrical clear peaks of heme iron reduction/oxidation [Figure 1(*b*)]. SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNTs do not have such CV peaks. The formal potential $E_{½} = (E_{ox} + E_{red})/2$ was –0.031 ± 0.007 V in 0.1 M PBS, pH 7.4 (under these conditions, the PAA segment was almost fully ionized). This value is consistent with well-known data for direct unmediated electron-transfer processes of cytochrome *c*. The ratio of anodic to cathodic peak currents was 1.02. These results confirmed that the anionic $PnBA_{100}$-*b*-PAA_{140}/MWCNT nanocomposite is a favorable matrix for cytochrome *c*. The protein binding to the nanocomposite layer is nondestructive, and it considerably facilitates reversible electron transfer. The cyclic voltammograms of SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT/cyt *c* at different scan rates and cathodic and anodic peaks currents are linearly proportional to the scan rate in a range from 5 to 100 mV s^{-1} (Figure S1, see Online Supplementary Materials); thus, the electrode reaction corresponds to a surface-controlled process (protein film voltammetry).

The surface concentration of electroactive cytochrome *c* Γ_0 was 10 nmol cm^{-2}, and the electron transfer rate constant (k_s) was calculated using the Laviron equation at a scan rate of 100 mV s^{-1} and ΔE_p > 200 mV.[13] The value of k_s for cytochrome *c* at SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT was 2 s^{-1}, which is comparable with this value for electrodes modified with nanocomposite materials such as macroporous Au film L-cystein/Au, poly-3-methylthiophene/CNT, or ionic liquid/CNT.[1,8,14] Our results indicate a strong interaction of positively charged cytochrome *c* with the almost fully ionized (at pH 7.4) carboxylic groups[10] of a polymeric matrix in SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT, mimicking the natural complex of this heme protein with anionic phospholipids.[4,9,10]

A dynamic range for cytochrome *c* biosensing was determined using SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT with a number of standard heme protein solutions featuring different concentrations of cytochrome *c*. The calibration curves were obtained by plotting the cathodic Faradaic current of DPV response or DPV peak area *vs.* cytochrome *c* concentration and fitted by a linear regression as DPV response = 0.62 [cytochrome *c*] with a correlation coefficient of 0.97 or as DPV peak area = 0.123 [cytochrome *c*] with a correlation coefficient of 0.93 (Figure S2). Therefore, using voltammetric DPV analysis, we determined cytochrome *c* in a range of 2–100 μM with a limit of detection (LOD) of 1.91 μM (S/N = 3). These electroanalytical characteristics are comparable with published data.[1]

The electroanalysis of cytochrome *c* by SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT was carried out in a range of positive potentials in order to detect not only the electrochemistry of heme as a prostetic group but also the electrooxidation of amino acids in the cytochrome *c* polypeptide chain as an irreversible electrochemical process.[15,16] In terms of electrochemistry, L-tyrosine (Tyr, Y) and tryptophan (Trp, W) have received the most attention because their electrocatalytic oxidation is a basis of label-free protein biosensors for the monitoring and detection of conformational changes in proteins, ligand/protein binding, and oxidative protein damage, phosphorylation, or nitration as an example of post-translational modification.[17] Nanomaterial-based electrodes can be used in the label-free electrochemical analysis of amino acids.[18]

Figure 2(*a*) shows the DVP response of cytochrome *c* oxidation at positive potentials as a single peak at +0.578 ± 0.011 V attributed to Tyr + Trp oxidation.[16] The calibration curves [Figure 2(*b*)] were fitted by a linear regression of $I_{p,c}$ ($I_{p,c}$ = 0.44 [cytochrome *c*]) with a correlation coefficient of 0.96. The LOD was 1.16 μM (S/N = 3), which was approximately two times lower than that for heme reduction near a 0 V potential.

We also observed a peak at +0.80 V, which can be attributed to the irreversible electrochemical transformation of a heme component (porphyrin ring) according to Novak *et al.*[16] Indeed, a diminished heme amplitude was measured for cytochrome *c* at a 0 V potential after the application of an anodic oxidation potential window (Figure S3) to confirm the electrochemical destruction of porphyrin itself.

Hence, using the SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT and applying a broad potential window, we were able to measure a panoramic CV or DPV, which reflects the overall electrochemical properties of cytochrome *c* under the given conditions. With this specific electrode modification, one can clearly see (*1*) the reduction and oxidation of the heme prostetic group at a near 0 V potential and (*2*) the electrooxidation of Tyr and Trp at +0.6 V and the irreversible electrochemical transformation of heme itself at +0.8 V (Figure 3).

Thus, a promising SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT electrode modification was developed, which allowed us for the first time to observe the electrochemistry of both parts of this particular protein, as we could access both the heme prosthetic group and the apoprotein by a single voltammetric scan using a broad potential window. Simultaneously we could examine the electrooxidative

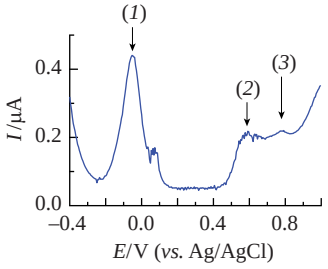


Figure 3 Panoramic DPV as an electrochemical fingerprint of cytochrome *c* deposited on the SPE/$PnBA_{100}$-*b*-PAA_{140}/MWCNT electrode surface: (*1*) response for 100 μM with oxidation/reduction of heme, (*2*) oxidation of the amino acids Tyr + Trp, and (*3*) electrooxidative destruction of heme.

properties of amino acids, such as Tyr and Trp, using the promising SPE/PnBA$_{100}$-*b*-PAA$_{140}$/MWCNT electrode modification. This multi-point detection of cytochrome *c* can be considered as an electrochemical fingerprint and a method for cytochrome *c* recognition and quantification in complex (bio)chemical analytes.

Generally, the electrochemical fingerprint of cytochrome *c* can characterize the redox status of heme iron and any modification of the polypeptide chain. This approach is label-free, and it avoids additional steps of affinity interactions with antibodies, aptamers, plastic antibodies (molecularly imprinted polymers), or protein redox partners such as cytochrome *c* reductase. Both the nitration and phosphorylation of Tyr residues in human cytochrome *c* are crucial functional alterations resulting in a defective apoptosome, altered redox potentials, the inhibition of apoptosis, or a decreased ability to activate and interact with caspases.[17,19] Therefore, the developed method of an electrochemical fingerprint of cytochrome *c* can be used for the detection of apoptotic events during chemotherapy, a search for new anticancer drugs, investigation of apoptosis mechanisms, or the detection of post-translational modifications of the protein polypeptide chain or the heme.[17,20]

This work was supported by the Russian Science Foundation (project no. 18-44-04011) and the Deutsche Forschungsgemeinschaft (SCHA1640/18-1). We are grateful to Dr. O. Colombani (Le Mans University, France) for the synthesis and characterization of the poly(*n*-butyl acrylate)$_{100}$-*block*-poly(acrylic acid)$_{140}$ diblock copolymer.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2020.05.012.

References

1 P. Manickam, A. Kaushik, C. Karunakaran and S. Bhansali, *Biosens. Bioelectron.*, 2017, **87**, 654.
2 Y. P. Ow, D. R. Green, Z. Hao and T. W. Mak, *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 532.
3 J. Yin and P. Miao, *Chem. – Asian J.*, 2016, **11**, 632.
4 R. Santucci, F. Sinibaldi, P. Cozza, F. Polticelli and L. Fiorucci, *Int. J. Biol. Macromol.*, 2019, **136**, 1237.
5 M. Pandiaraj, T. Madasamy, P. N. Gollavilli, M. Balamurugan, S. Kotamraju, V. K. Rao, K. Bhargava and C. Karunakaran, *Bioelectrochemistry*, 2013, **91**, 1.
6 C. Guo, J. Wang, X. Chen, Y. Li, L. Wu, J. Zhang and C. Tao, *Sensors*, 2019, **19**, 40.
7 M. Hasanzadeh, N. Shadjou and M. de la Guardia, *Trends Anal. Chem.*, 2017, **93**, 199.
8 M. Zhang, J. Zheng, J. Wang, J. Xu, T. Hayat and N. S. Alhardi, *Sens. Actuators, B*, 2019, **282**, 85.
9 V. E. Kagan, H. A. Bayir, N. A. Belikova, O. Kapralov, Y. Y. Tyurina, V. A. Tyurin, J. Jiang, D. A. Stoyanovsky, P. Wipf, P. M. Kochanek, J. S. Greenberger, B. Pitt, A. A. Shvedova and G. Borisenko, *Free Radical Biol. Med.*, 2009, **46**, 1439.
10 O. Colombani, M. Ruppel, F. Schubert, H. Zettl, D. V. Pergushov and A. H. E. Müller, *Macromolecules*, 2007, **40**, 4338.
11 L. V. Sigolaeva, T. V. Bulko, M. S. Kozin, W. Zhang, M. Köhler, I. Romanenko, J. Yuan, F. H. Schacher, D. V. Pergushov and V. V. Shumyantseva, *Polymer*, 2019, **168**, 95.
12 V. V. Shumyantseva, L. V. Sigolaeva, L. E. Agafonova, T. V. Bulko, D. V. Pergushov, F. H. Schacher and A. I. Archakov, *J. Mater. Chem. B*, 2015, **3**, 5467.
13 E. Laviron, *J. Electroanal. Chem.*, 1979, **101**, 19.
14 T. M. Yawitz, K. S. Patterson, B. X. Onkst, F. Youmbi and R. A. Clark, *J. Electroanal. Chem.*, 2018, **828**, 59.
15 V. Brabec, *Stud. Biophys.*, 1980, **81**, 93.
16 D. Novak, M. Mijovic, A. Pavicevic, M. Zatloukalova, L. Hernychova, M. Bartosik and J. Vacek, *Bioelectrochemistry*, 2018, **119**, 136.
17 Y.-W. Lin, *Arch. Biochem. Biophys.*, 2018, **641**, 1.
18 X. Yu, Z. Mai, Y. Xiao and X. Zou, *Electroanalysis*, 2008, **20**, 1246.
19 R. Mohan and S. K. Atreja, *Cryobiology*, 2015, **70**, 253.
20 V. V. Shumyantseva, E. V. Suprun, T. V. Bulko and A. I. Archakov, *Biosens. Bioelectron.*, 2014, **61**,131.

Received: 15th November 2019; Com. 19/6057