

Direct Electrochemistry of Heme Proteins on Electrodes Modified with Didodecyldimethyl Ammonium Bromide and Carbon Black

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Abstract

A novel matrix based on commercially available carbon black (CB) N220 and didodecyldimethyl ammonium bromide (DDAB) was shown to be a reliable support for direct electron transfer reactions between screen printed electrode (SPE) and Fe(III)-heme proteins. Cytochrome *c* (cyt *c*), myoglobin (Mb), horseradish peroxidase (HRP) and cytochromes P450 (CYP 51A1, CYP 3A4, CYP 2B4) generated well-shaped cyclic voltammograms on SPE/CB/DDAB electrodes (both in solution and in immobilized state). The attractive performance characteristics of CB modified electrodes are advantageous over single-walled carbon nanotubes (SW CNT) based ones. The achieved direct electrochemistry of heme proteins on CB/DDAB-modified electrodes provided successful elaboration of the immunosensor for cardiac Mb. The immunosensor showed applicability for diagnostics of myocardial infarction displaying significant difference in cardiac Mb content of human blood plasma samples taken from the corresponding patients.

Keywords: Carbon black, Cardiac myoglobin, Didodecyldimethyl ammonium bromide, Direct electron transfer, Heme protein

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1 Introduction

In supporting living organisms on the earth, heme proteins play a vital role in various life processes. These include the transport and storage of molecular oxygen by hemoglobin (Hb) and by Mb, the transfer of electrons from respiratory substrates by cytochromes and the terminal oxidation with O₂ by cytochrome *c* oxidase, the decomposition of hydrogen peroxide by catalase, the oxidation of organic substances with H₂O₂ by peroxidase, the hydroxylation of organic substances through dioxygenation by cytochrome P450, the synthesis of nitric oxide from L-Arg by NO synthase, and so forth. As a matter of fact, all these different functions are primarily based upon the oxidation–reduction properties of the heme iron in itself [1]. Based on their unique catalytic and electrochemical properties, the heme proteins are finding an increasing application in biosensor construction for the detection of different biologically active species. On the other hand, determination of heme proteins (such as Hb, Mb) in very small concentrations in human biological fluids is still a highly topical issue.

Heme proteins based biosensors operating through direct electron transfer reactions (which electrons being directly transmitted between the electrode and the heme iron) belong to the third-generation biosensors [2,3]. However, such electrocatalytic reactions can only be ac-

complished by using special electrode surface modifiers [4–6]. Efficiency of direct electron transfer between the electrode and the heme depends on electrode material, electrode surface modification and proper orientation of the protein active center on the electrode.

Carbon materials, owing to the availability of different conducting allotropes, the good thermal and electrochemical stability, and a wide range of carbon microstructures of varying hardness, cost, and reactivity, are widely used in electrochemistry for electrode construction and modification. “Classical” carbon materials are based on graphite, glassy carbon, and CB. Recently developed materials can be subdivided into microfabricated carbon, conducting diamond, fibers and nanotubes, and carbon composites. The propensity of carbon to adsorb molecules from solution and the presence of surface oxides permit electrocatalytic reactions on carbon electrodes that are weaker or absent on metal electrodes. The Fe(III)/(II) redox reaction strongly depends on the presence of surface oxides on carbon and is inhibited significantly if they are absent or obscured by adsorbates. The Fe-O bond in hydrated Fe(III) changes length upon reduction, and this process is facilitated by transient interactions with surface oxides [7].

Carbon materials are among the most common in bioelectrochemistry due to their (i) electrochemically inert surface and (ii) high sorption ability. CB based electrodes,

graphite rods, edge plan pyrolytic graphite, carbon filament materials and even screen printed carbon electrodes are used as support for bioelectrocatalytic systems. Prior to enzyme immobilization, hydrophobic materials (carbon filament, CB) have to be hydrophilized chemically or electrochemically, with subsequent thorough washing – to prevent protein coagulation [3].

CB is virtually pure elemental carbon in the form of colloidal particles that are produced by incomplete combustion or thermal decomposition of gaseous or liquid hydrocarbons under controlled conditions (www.carbon-black.org). While diameters of primary particle (near spherical building blocks of CB) are generally in the 10–300 nm range, CB products as placed into commerce (the final products) are agglomerates, which are much larger in size (100–1000 nanometers in diameter).

In late 70s of the XX century the direct bioelectrocatalysis was discovered using CB electrode for the enzyme laccase immobilization [8]. In this decade, CB showed itself as an advantageous carbon material in analytical electrochemistry due to its low cost manufacturing, simple treatment and electrocatalytic properties towards many biologically active compounds such as ascorbic acid [9,10], dopamine [10], reduced form of nicotinamide adenine dinucleotide (NADH) [11,12], benzoquinone [11,12], epinephrine [11,12], cysteine and thiocholine [13], some heavy metals (Cd(II), Pb(II)) [10] and hydrogen peroxide [10,12]. CB modification of the sensors provides an improvement in terms of the low peak-to-peak separation and an increase of peak current intensity of the compound tested. A sensor based on polyethylene-graphitized CB as an electrochemical detector in a high-performance liquid chromatography (HPLC) analytical system was developed. It showed a higher signal-to-noise ratio for several compounds tested such as phenol, chlorophenols and hydroquinone [14]. The suitability of CB for biosensor applications was demonstrated with tyrosinase to produce a biosensor that was challenged in amperometric mode with catechol. The highest sensitivity, equal to 625 nA/mM, coupled with the lowest detection limit of 0.008 mM, was observed [9]. Reagentless enzyme biosensors based on the quinoxaline protein alcohol dehydrogenase and graphitized CB, suitable for determination of ethanol, glucose and hydrogen peroxide (with electrocatalytic oxidation taking place at 0.1–0.4 V vs. Ag/AgCl), were designed using screen-printing technique by Razuviene et al. [15]. The direct electrochemical reaction of Hb adsorbed on the surface of CB with an underlying glassy carbon electrode was first reported by Ma and co-workers [16]. CB powders were dispersed thoroughly in 0.1 wt.% cetyltrimethylammonium bromide (CTAB) solution which served as a dispersant to prevent the CB powders aggregation following ultrasonication. The Hb molecules were adsorbed on the surface of CB powders during mixing. The immobilized Hb retained its bioelectrocatalytic activity for the reduction of H₂O₂.

Liquid crystal surfactant films for electrochemical catalysis were thoroughly studied by Rusling's group [17]. It

was shown that standard electron-transfer rate constants for the Fe(III)/Fe(II) redox couple of Mb were about 1000-fold higher in liquid crystal films of didodecylmethyl ammonium bromide on pyrolytic graphite electrodes than in aqueous solutions [18]. Mb retains the heme iron and near-native conformation in DDAB films [19]. The electron transfer rate of the Hb heme's Fe(III)/Fe(II) couple was much greater in DDAB films than it was on bare edge-plane pyrolytic graphite electrodes or electrodes modified with mediators with Hb in solution [20]. Electronic absorbance linear dichroism showed that Hb and Mb are preferentially oriented in the films [19,20]. Therefore, surfactants (DDAB, CTAB) provide good adsorption of heme proteins and serve not only as dispersants to prevent the powder aggregation but also as catalysts of electrochemical reactions.

In this work, a novel matrix based on commercially available CB N220 and surfactant DDAB was tested for registration of direct electrochemical reactions of heme proteins. Several heme proteins from different classes were chosen as the objects of this investigation: cyt *c*, Mb, HRP and CYPs. For all proteins tested the well-shaped reduction and oxidation peaks were registered by cyclic (CV) and square wave voltammetry (SWV). The characteristics of CB as an electrode surface modifier were advantageous over SW CNT ones. Based on direct electrochemistry of heme proteins on CB-modified electrodes, the immunosensor for cardiac Mb (cMb) detection was developed. The ability of this newly-obtained device to detect myoglobin in human plasma may find an application in diagnostics of acute myocardial infarction (AMI).

2 Experimental

2.1 Apparatus and Electrochemical Measurements

Cyclic voltammetry and square wave voltammetry measurements were performed using an Autolab electrochemical system PGSTAT-12 (Eco Chemie, Utrecht, The Netherlands) equipped with GPES software (Eco Chemie, Utrecht, The Netherlands). Electrochemical impedance spectroscopy (EIS) measurements were carried out in the same electrochemical cell with a PC-controlled Autolab and FRA software (Eco Chemie, Utrecht, The Netherlands). A sinusoidal voltage perturbation of 10 mV amplitude was applied over the frequency range 10 kHz to 0.01 Hz with 10 measurement points per frequency decade. For the fitting of the data obtained by EIS, Zviews software (Scribner Associates, Inc.) has been used with the Randles equivalent circuit [21]. Screen-printed graphite electrodes were home made by a 245 DEK (Weymouth, UK) screen-printing machine. Graphite based ink (Electrodag 423 SS) from Acheson (Milan, Italy) for printing the working and counter electrode, and silver ink (Electrodag 477 SS) for the pseudo-reference electrode were used. The substrate was a flexible polyester film (Autostat HT5) obtained from Autotype Italia

(Milan, Italy) by using the procedure and materials described earlier [12]. The electrodes were produced in foils, each containing 48 electrodes. The diameter of the working electrode was 0.3 cm resulting in a geometric area of 0.071 cm². All potentials were referred to the Ag screen-printed pseudo-reference electrode. All electrochemical experiments were carried out at room temperature (20–25 °C) in a 100- μ L drop put onto the SPE so as to cover all 3 electrodes.

2.2 Chemicals and Proteins

The following heme proteins were used: equine myoglobin from skeletal muscles (Serva); horse heart cytochrome *c* (Merck); horseradish peroxidase (HRP, Sigma, 1000 units/mg solid, using ABTS); cytochrome P450 51A1 (human, recombinant, Gene ID: 1595 CYP 51A1, 165 μ M, in 300 mM potassium–phosphate buffer, pH 7.2, containing 20% glycerol, 0.2% CHAPS and 1 mM DTT); cytochrome P450 3A4 (human, recombinant, Gene ID: 1576 CYP 3A4, 165 μ M, in 550 mM potassium–phosphate buffer, pH 7.2, containing 20% glycerol, 0.2% CHAPS and 1 mM DTT). CYP 51A1 and CYP 3A4 were kindly presented by Prof. S. A. Usanov (Institute of Bioorganic Chemistry, Minsk, Belarus). Cytochrome P450 2B4 (100 μ M) was isolated from the microsomal fraction of rabbit liver induced by phenobarbital and purified as described in [22]. Concentration of P450 2B4 was determined by formation of a complex of the cytochrome P450's reduced form with carbon monoxide using the extinction coefficient $\epsilon_{450} = 91 \text{ mM}^{-1} \text{ cm}^{-1}$ [23]. Mouse anti-human myoglobin, cardiac (anti-cMb, mass concentration 1.05 mg/mL, M9800–16A) was purchased from USBiological.

Plasma samples were obtained from healthy donors and patients with AMI. Informed signed consent was obtained from each of the patients. Plasma was collected after centrifugation of blood with EDTA as anticoagulant for 10 min at 3000 rpm. Cardiac myoglobin concentration as indicator of AMI was determined in each sample with the bench-top lateral flow immunoassay RAMP (Response Biomedical Corp) according to the instruction. The samples from the bank of plasma of healthy donors and patients with AMI with cMb mass concentrations 190 ± 19 and 350 ± 35 ng/mL were used for testing [24].

Salts (KH₂PO₄, KCl, NaCl, potassium ferrocyanide trihydrate, potassium hexacyanoferrate (III)) were from Sigma-Aldrich and Carlo Erba reagenti. Didodecyltrimethylammonium bromide was from Sigma-Aldrich. Solvents: Chloroform was from Carlo Erba reagenti and *N,N*-dimethylformamide (DMF) was from Merck.

CB N220 was obtained from Cabot Corporation (Ravenna, Italy). As reported by the manufacturer, CB particles used had a diameter comprised between 19 and 29 nm with a surface area of 124 m²/gas measured by BET method (N₂ absorption). Single wall carbon nanotubes were from NanoLab (SW CNT, COOH functional-

ized, lot. PD30L5–20-COOH). All the chemicals were of analytical grade.

2.3 Solutions

Electrochemical experiments were carried out in phosphate buffer (PB) containing: 0.1 M KH₂PO₄ + 50 mM NaCl, pH 6.5. 5 mM K₃[Fe(CN)₆] in 0.1 M KH₂PO₄ + 1 M KCl (pH 7.4) was used to evaluate the efficient surface area of the working electrode. 5 mM K₃[Fe(CN)₆] + 5 mM K₄[Fe(CN)₆] in 0.05 M phosphate buffer + 0.1 M KCl was used as supporting electrolyte for electrochemical impedance spectroscopy. Heme proteins (Mb and cyt *c*) were tested directly or immobilized onto the electrode surface from 500 μ M or 1 mM stock solutions in PB. HRP (2160 U/mL) was dissolved in citrate buffer, pH 5.0. For electrode modification 0.1 M solution of DDAB in chloroform was used.

2.4 Preparation of SW CNT Dispersion (Mass Concentration 1 mg/mL)

SW CNT were oxidized according to the procedure described earlier in [25]. For oxidation 4 mg of SW CNT were weighted into a glass bottle, then 1 mL of HNO₃ (conc. 65%) and 3 mL of H₂SO₄ (conc. 95%) were added. Resulting mass concentration of SW CNT was 1 mg/mL of the acid mixture, and the acid ratio HNO₃:H₂SO₄ was 1:3. The bottle was hermetically closed with a cap, then put into ultrasonic bath and ultrasonicated for 6 hours, at 50 °C. After sonication, the resulting mixture was split up into 1.5-mL eppendorfs, with 0.5 mL of the mixture being added to each eppendorf (the resulting content was 0.5 mg of SW CNT per eppendorf). The eppendorfs were centrifuged for 20 min at a speed of 12000 rpm. After centrifugation the acidic solution was gently removed from the eppendorfs with a micropipette and the nanotubes cleaned with distilled water. To do that, 1 mL of water was added to each eppendorf, centrifuged for 20 min at the speed 12000 rpm and removed gently with a micropipette. The washing procedure was repeated 3 times. After removing the water in the last iteration of the cleaning, the eppendorfs with nanotubes were air-dried. The oxidized nanotubes were dispersed in DMF-water solution at the ratio 1:1 – by adding 0.5 mL of the solution into each eppendorf – and sonicated at 59 KHz for 120 min at 50 °C. The resulting mass concentration of SW CNT was 1 mg/mL.

2.5 Preparation of CB Dispersion (Mass Concentration 1 mg/mL)

To prepare the CB dispersion, 10 mg of freshly prepared CB powder was added to 10 mL of DMF - water mixture (1:1) and then sonicated for 60 min at 59 KHz.

2.6 SPE Modification by CB or SW CNT

The SPE was modified with CB (SPE/CB) or with CNTs (SPE/CNT) as follows: 6 μL -volume of the CB or CNT dispersions was pipetted onto the working electrode surface of the SPE by three steps of 2 μL each. After each deposition, the solvent was allowed to evaporate.

2.7 Preparation of Electrochemical Biosensors (SPE/CB or CNT/DDAB/(Heme Protein))

To the surface (bare or modified by CB or SW CNT) of the working graphite electrode 0.25 μL of 0.1 M DDAB were added; after evaporation of chloroform, heme protein (Mb, cyt *c*, cytochrome P450 or HRP) was immobilized onto the SPE/CB (CNT)/DDAB: 0.25 μL of protein solution was dropped onto the electrode surface and kept at 4 °C overnight. SPE/DDAB, SPE/CB/DDAB or SPE/CNT/DDAB were used in control experiments or experiments with protein solutions.

2.8 Preparation of Electrochemical Immunosensors (SPE/CB/DDAB/anti-cMb)

Immunosensors based on CB modified electrodes were prepared according to the principal circuit described in [24,26]. 0.25 μL of 0.1 M DDAB were deposited on the surface of SPE/CB (6 μL); after evaporation of chloroform, the antibodies were immobilized onto the SPE/DDAB: 0.25 μL of 105 ng/ μL anti-cMb solution in PB (pH 6.5) were dropped onto the electrode surface and kept at 4 °C overnight.

2.9 Mb Determination Procedure

The 0.25- μL sample of Mb in PB (or undiluted human blood plasma) was loaded onto the sensor surface (i.e. onto SPE/CB/DDAB, SPE/DDAB or SPE/CB/DDAB/anti-cMb, SPE/DDAB/anti-cMb). Sensor was allowed to stay for 15 min at 37 °C. Then electrochemical measurements were carried out at 25 °C. The 100- μL drop of PB was put on the electrode surface to cover all three electrodes (working, counter and reference); 5 min after, the electrochemical registration of the signal (cathodic peak area or current at $E_{\text{max}} \approx -250$ mV) was carried out by SWV. The following experimental parameters of SWV were used: initial potential, $E_i = 0.1$ V; end potential, $E_e = -0.6$ V; pulse height, $\Delta E_p = 20$ mV; step potential, $\Delta E_s = 5$ mV; square wave frequency, $f = 10$ Hz.

3 Results and Discussion

3.1 Ferricyanide/Ferrocyanide Redox Couple on SPEs Modified with CB or SW CNT

Examination of electron transfer kinetics on SPE/CB and SPE/SW CNT was performed using 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in phosphate buffer (pH 7.4) by applying cyclic voltammetry at different scan rates in the range between 0.005 and 0.05 V/s. Figure 1 illustrates CVs of ferricyanide ion reduction and ferrocyanide oxidation on bare SPE and SPEs modified with CB and SW CNT (6 μL of dispersion/electrode).

The electrochemical characteristics of the ferricyanide/ferrocyanide redox couple on SPE and SPEs modified with CB or SW CNT are summarized in Table 1. Modifi-

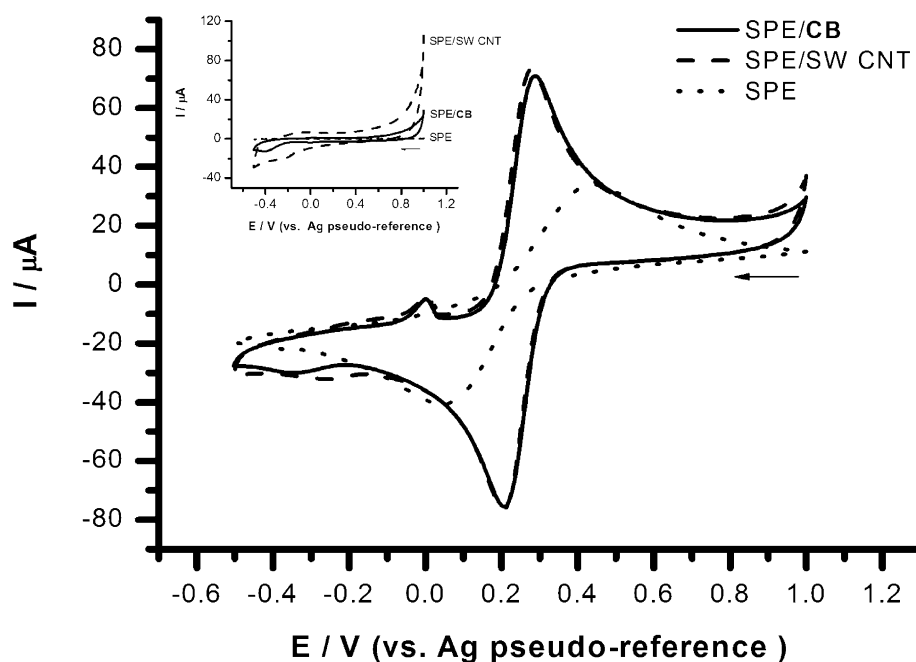


Fig. 1. CVs of 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ (in 0.1 M $\text{KH}_2\text{PO}_4 + 1$ M KCl , pH 7.4) using bare SPE, SPE/CB and SPE/SW CNT. Inset: Baselines for bare SPE, SPE/CB and SPE/SW CNT. Potential scan rate is 50 mV/s, from 1000 to -500 mV.

cation of the SPE surface with CB or SW CNT significantly increased the rate of the Fe(III)/Fe(II) redox process. SPEs modified with 2, 4 and 6 μL of CB or SW CNT dispersions were tested. For both dispersions the 6- μL volume was found saturated for SPE modification (data not shown). Following CB or SW CNT modifications, the effective surface area of electrodes was enlarged twofold (6 μL of dispersion/electrode). Based on Randles–Sevcik's equation with the diffusion coefficient $D = 5.9 \times 10^{-5} \text{ cm}^2/\text{s}$ [27], the effective surface areas (A) of bare SPE and SPEs modified with CB and SW CNT were estimated (Table 1). As is clear from Figure 1, the ferricyanide reduction–ferrocyanide oxidation peaks on SPE/CB and SPE/SW CNT are almost equal (the equal amounts (6 μL) of SW CNT or CB dispersion were put on SPEs), but the baseline for SPE/CB appeared to be much lower than that for SPE/SW CNT. A small peak-to-peak separation (ΔE_p) with increasing scan rates was observed for both SPE/CB and SPE/SW CNT (Table 1). Such behavior of the ferricyanide ions suggests a quasi reversible mechanism of electrochemical reaction. The formal potential (E°) and the peak-to-peak separation for the ferri/ferrocyanide redox couple on SPE/CB were nearly equal to those obtained on SPE/SW CNT (with $\nu = 50 \text{ mV/s}$) (Table 1). The current of the anodic and cathodic peaks increased linearly with the square root of the scan rate (5–50 mV/s), indicating that the process was diffusion controlled for all electrodes under study (data not shown). Based on Nicholson's theory [28], the heterogeneous rate constants (k°) for the ferricyanide/ferrocyanide redox couple on bare and modified electrodes were calculated – proceeding from the assumption that $D_{\text{O}} = D_{\text{R}} = 5.9 \times 10^{-5} \text{ cm}^2/\text{s}$ and an electrochemical transfer coefficient $\alpha = 0.5$, since the $I_{\text{p,a}}/I_{\text{p,c}}$ value is very close to unity (Table 1).

Table 1. Electrochemical characterization of bare SPE, SPE/CB and SPE/SW CNT by using the ferricyanide/ferrocyanide redox couple: 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ in phosphate buffer (pH 7.4); CV, from 1000 to -500 mV ; scan rate, 50 mV/s .

Characteristics	SPE	SPE/CB	SPE/SW CNT
A (cm^2)	0.016 ± 0.002 [a]	0.032 ± 0.003	0.032 ± 0.003
E° (V)	0.244	0.246	0.245
ΔE_p (V)	0.371	0.078	0.071
k° (cm/s)	$1.0 \pm 0.1 \times 10^{-2}$	$1.4 \pm 0.1 \times 10^{-2}$	$1.9 \pm 0.2 \times 10^{-2}$

[a] The geometric area is 0.071.

The more intensive electron transfer of the ferricyanide/ferrocyanide redox couple with modified SPEs was again confirmed by using EIS. The spectra obtained at the open circuit potential were plotted as Nyquist plot ($-Z_i$ vs. Z_r) in the complex plane (data not shown). The relevant parameter in these experiments is the charge transfer resistance, R_{ct} , which represents the difficulty of electron transfer of a ferro/ferricyanide redox probe between the solution and the electrode, providing information about the electrochemical interphase. Fitting of spec-

tra was done using a Randles equivalent electrical circuit which is comprised of the electrolyte resistance, R_s , in a series with a parallel combination of R_{ct} , Z_w (diffusion of the analytes in solution) and constant phase element (CPE). It was necessary to introduce the CPE, because of the non-homogeneous surfaces of both the bare and modified SPEs. Using the SPE/CB, we have obtained the R_{ct} value equal to $222 \pm 27 \Omega$ instead of $38 \pm 2 \Omega$ in case of SPE/SWCNT; the both values were much lower than the R_{ct} value for bare SPE ($4004 \pm 6 \Omega$) reported in our previous paper [12]. Clearly, modification of the working electrode surface with a layer of CB or SW CNT can markedly promote the electrochemical reaction of the Fe(III)/Fe(II) couple.

Electrochemical studies of ferricyanide ions in solutions have shown that analytical characteristics of the electron transfer for SPE/CB are comparable to those for SPE/SW CNT and, also, to the earlier reported data on SW CNT and MW CNT modified electrodes [27,29]. Moreover, as was shown earlier by Compton group, carbon nanotubes and graphite powder modified basal plane pyrolytic graphite demonstrated similar electrocatalytic behavior toward NADH, epinephrine and norepinephrine [30]. As is known from our previous studies and literature data, the list of analytes, in relation to which CB exhibited the electrocatalytic properties (ascorbic acid, dopamine, NADH, benzoquinone, epinephrine, cysteine and thiocholine [9–13]), correlates with the list of compounds for CNT-modified electrodes [31], which also confirms the similar electrocatalytic behavior of CB and CNT on the electrode surface. Therefore, like other carbon materials, CB provides enlargement of the effective surface area of working electrode and exhibits high-quality electrochemical properties with enhanced currents and reduced peak-to-peak separations upon voltammetry. One very important CB advantage is a large number of edge-plane/defect sites in its structure – as was shown for the open ends of nanotubes, for the basal plane highly ordered pyrolytic graphite [32] and, also, for the SPE surface [33]. Raman spectrometry of CB revealed the presence of D and G bands, while the I_D/I_G ratio – a measure of the number of disordered and defect sites – was equal to 0.96: this value is comparable to the literature data reported for CNT [11].

3.2 Direct Electrochemistry of Heme Proteins on DDAB/CB/SPE

Reduction-oxidation CVs for Mb and cyt *c* solutions were registered with SPE, SPE/CB, SPE/DDAB and SPE/CB/DDAB. Reduction of Fe(III)-heme of Mb was not observed on the bare SPE, and the reduction-oxidation process of the Fe(III)-heme of cyt *c* was very slow. The modification of the electrode surface with CB increased the baseline current but did not significantly change the signal-to-noise ratios and rates of the electron transfer processes. In agreement with Rusling's work [18], to promote direct electron transfer between Fe(III)-heme and

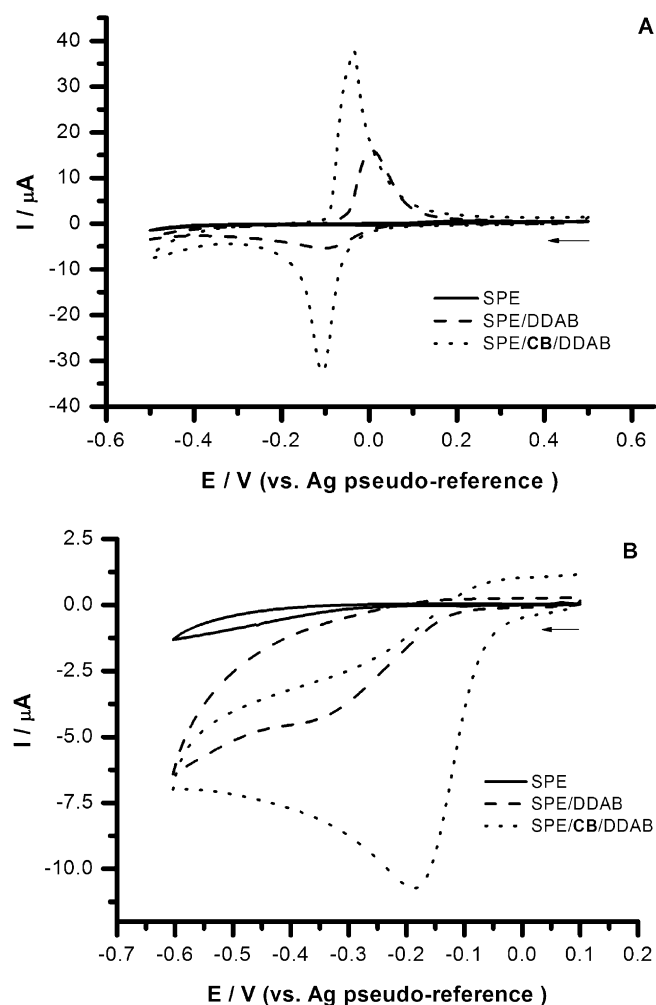


Fig. 2. CVs of 500 μM cyt *c* (A) and 500 μM Mb (B) in PB, pH 6.5, using SPE, SPE/DDAB, SPE/CB/DDAB. Potential scan range is from 100 to -600 mV for Mb and from 500 to -500 mV for cyt *c*; scan rate, 50 mV/s.

the electrode surface, DDAB film was adsorbed on CB/SPE. As shown in Figure 2, CB layer dramatically increased the reduction and oxidation peaks of Fe(III)-heme on SPE/CB/DDAB. With the SPE/CB/DDAB, cyt *c* in solution demonstrated a more reversible redox process than with SPE/DDAB (Figure 2A).

Electrochemical reaction of cyt *c* can be expressed according to Equation 1:

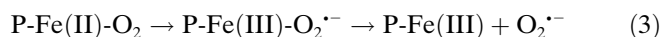
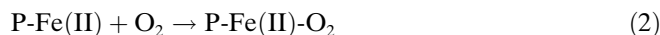


where P-Fe(III)/P-Fe(II) is heme protein in Fe(III)/Fe(II)-state.

With SPE/CB/DDAB, $E^{\circ'}$ for Fe(III)/Fe(II)-heme of cyt *c* was found to be 71 ± 5 mV, and ΔE_p was equal to 68 ± 5 mV vs. $E^{\circ'} = -45 \pm 5$ mV and $\Delta E_p = 103 \pm 5$ mV for SPE/DDAB – with the scan rate, in both cases, being equal to 50 mV/s. The current of the anodic and cathodic peaks of cyt *c* increased linearly with the square root of the scan rate between 0.005–0.1 V/s, indicating that the

process was diffusion controlled: $I_{p,c}$ (μA) = $11.8 \pm 0.7 - 184 \pm 9 v^{1/2}$ (V/s) $^{1/2}$ and $I_{p,a}$ (μA) = $-11 \pm 1 + 211 \pm 13 v^{1/2}$ (V/s) $^{1/2}$ ($R = 0.997$, $n = 6$, $RSD = 10\%$).

The CVs of Mb solution are presented in Figure 2B. With SPE/CB/DDAB, Mb had two well defined reduction and oxidation peaks. Mb (after one-electron reduction (Equation 1)) in Mb-Fe(II)-state fast binds oxygen (Equation 2) and autoxidized (Equation 3):



With SPE/CB/DDAB, $E^{\circ'}$ for the Fe(III)/Fe(II)-heme of Mb was found to be -137 ± 5 mV, and ΔE_p was equal to -101 ± 5 mV, with the scan rate being 50 mV/s. The current of the cathodic peak of Mb reduction on SPE/CB/DDAB and SPE/DDAB increased linearly with the square root of the scan rate. The slopes of curves $I_{p,c}$ vs. $v^{1/2}$ for the cathodic peaks of Mb reduction from their solutions on SPE/CB/DDAB and SPE/DDAB were found equal to $-(3.4 \pm 0.2) \times 10^{-5}$ (0.005–0.1 V/s) and $-(2.2 \pm 0.1) \times 10^{-6}$ (0.005–0.03 V/s) A/(V/s) $^{1/2}$, respectively. From the above data it follows that CB promotes electro-reduction process of cyt *c*-Fe(III) and Mb-Fe(III) in solutions.

In the course of biosensors development, CB/DDAB was tested as an immobilization matrix for different heme proteins: cyt *c*, Mb, HRP, CYP 3A4, CYP 2B4, and CYP 51A1. Direct electrochemistry of these heme proteins immobilized on SPE/CB/DDAB was observed. Heme proteins (P-Fe(III)) which formed a complex with oxygen in P-Fe(II)-state (Mb, HRP, CYPs) being immobilized on SPE/CB/DDAB demonstrated a similar behavior that of Mb in solution: the reduction peak increased and appeared to be more narrow, the oxidation peak also increased and became well defined compared to SPE/DDAB. Figure 3 demonstrates the reduction-oxidation CVs of HRP and CYP 2B4 immobilized on SPE/CB/DDAB. Electrochemical characteristics of direct electron transfer reactions of different heme proteins immobilized on SPE/CB/DDAB are presented in Table 2.

In case of cyt *c*, CB did not improve the redox process (Equation 1) characteristics of this protein: the signal-to-noise ratio for SPE/DDAB/cyt *c* did not change significantly compared to SPE/CB/DDAB/cyt *c*.

The separation between CV reduction and oxidation peaks increases with increasing scan rate, because of limiting electron-transfer kinetics. The ΔE_p values were used to estimate the rate constants for electron transfer between electrodes and cyt *c* by the method of Laviron [34]. Apparent surface electron transfer rate constants (k_s) for SPE/DDAB/cyt *c* and SPE/CB/DDAB/cyt *c* were calculated given that $\alpha = 0.5$, the scan rate 50 mV/s, and $\Delta E_p(\text{Fe(III)/Fe(II)}) < 200$ mV, they were estimated as $0.93 \pm 0.09 \text{ s}^{-1}$ and $1.22 \pm 0.12 \text{ s}^{-1}$, respectively.

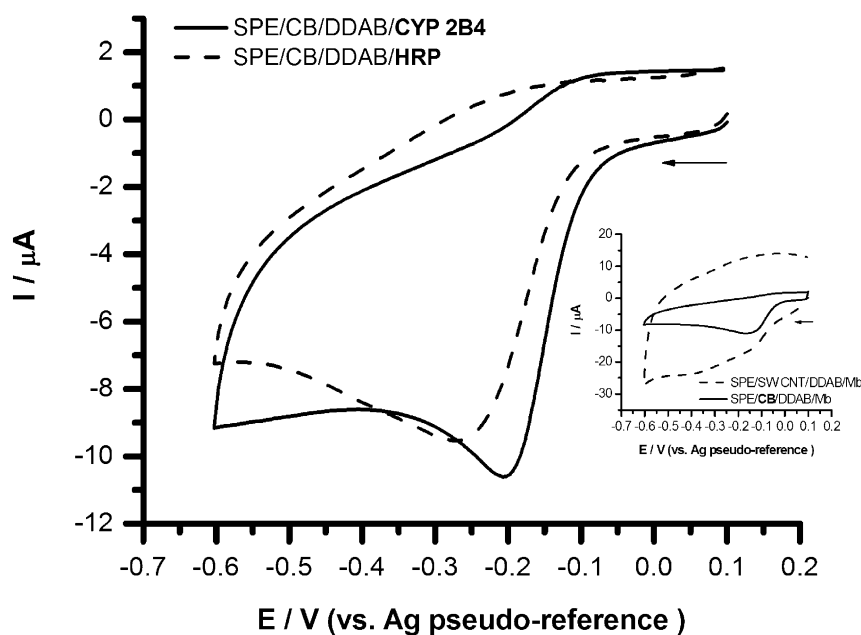


Fig. 3. CVs of SPE/CB/DDAB/imm. protein: 100 μM CYP 2B4 (—) and 50 μM HRP (---). Insert: CVs of 1mM Mb immobilized on: SPE/CB/DDAB (—) and SPE/SW CNT/DDAB (---). 0.25- μL aliquots of 0.1 M DDAB and heme proteins' solutions were taken. Potential range was from 100 to -600 mV, scan rate, 50 mV/s. PB, pH 6.5.

Table 2. Electrochemical characteristics of the direct electron transfer reactions for cyt *c* (1 mM), Mb (1 mM), HRP (50 μM), CYP 3A4 (165 μM), CYP 2B4 (100 μM), and CYP51A1 (165 μM) immobilized on DDAB/CB/SPE. Aliquots of heme proteins' solutions were 0.25 μL per electrode. CV, potential range from 100 to -600 mV; 50mV/s. PB, pH 6.5.

Heme protein	$E_{p,c}$ (V)	$E_{p,a}$ (V)	ΔE_p (V)	$E^{o'}$ (V)
Cyt <i>c</i>	-0.032	+0.020	0.052	-0.006
Mb	-0.186	-0.048	0.138	-0.117
HRP	-0.267	-0.159	0.109	-0.213
CYP 51A1	-0.197	-0.073	0.124	-0.135
CYP 3A4	-0.267	-0.130	0.137	-0.199
CYP 2B4	-0.205	-0.110	0.095	-0.158

The k_s obtained for cyt *c* immobilized on SPE/CB/DDAB ($1.22 \pm 0.12 \text{ s}^{-1}$) is similar to that reported earlier for Hb adsorbed on the CB surface with an underlying glassy carbon electrode (1.02 s^{-1}) [16]. In this latter work the Hb molecules were adsorbed on the surface of CB powders during mixing. It is to be noted that CB powders were dispersed in 0.1 wt% solution of CTAB which, acting as a dispersant, allowed to prevent the CB powders aggregation following ultrasonication. In the cyclic voltammogram of the Hb-CTAB/GC electrode, no redox peaks were observed. Conversely, a pair of well-defined and nearly symmetrical redox peaks was obtained at the Hb-CB/GC electrode. Based on CV data, Ma and coworkers [16] concluded that direct electron transfer reaction of Hb is not promoted by the surfactant CTAB or Nafion but is rather promoted by CB due to the presence of the oxygen-containing groups and many active sites on the surface. However, based on researches by Rusling's group [17-20] and our above-cited results (see Figure 2) it

was inferred that surfactants (DDAB, CTAB) play an important role in direct electron transfer reactions of heme proteins. Without DDAB, CB did not exhibited any catalytic properties towards heme proteins. It may well be that DDAB is able to hydrophilize hydrophobic CB and, hence, serves as a bridge between hydrophilic protein molecules and hydrophobic electrode surface.

The results obtained with the DDAB/CB matrix for heme proteins' direct electron transfer are in close agreement with those reported earlier by C. Cai et al. [35]. Authors demonstrated that the direct electron transfer rate of Hb was significantly enhanced after immobilization of this heme protein onto the surface of CNT dispersed in the solution of the surfactant CTAB.

Based on: (i) the similar behavior of CB and CNT towards electroactive substances [31] and (ii) the electrochemical catalytic properties of surfactant films (DDAB, CTAB) exhibited towards heme proteins [17,18], the CB/DDAB matrix was compared with SW CNT/DDAB with respect to direct electron transfer registration of immobilized Mb and cyt *c*. However, it is evident from the insert to Figure 3 that in air (i.e. without deoxygenation of solution) SW CNT in combination with DDAB is not suitable for heme proteins' redox activity registration and determination because of the unsatisfactory signal-to-noise ratio. It appears that with respect to electrocatalytic properties and the signal-to-noise ratio CNT, as an electrode surface modifier, yields to CB.

3.4 Mb Calibration Curve

Mb participates in human respiration both as an oxygen store and as an entity facilitating oxygen diffusion, there-

by supporting cellular respiration in cardiac and skeletal muscle tissues. In addition to conventional functions, it can possibly act, as has been recently proposed, as an intracellular scavenger of nitric oxide (NO) to protect mitochondrial cytochrome *c* oxidase from its inhibition by NO [1]. In healthy organism, in the absence of inflammation or injury of muscle tissues, myoglobin does not release into the circulation. Mb detection is very important for express diagnosis of acute myocardial infarction (cardiac isoform), crush syndrome, hypoxia neonatorum, overtraining of sportsmen, as well as in forensic medicine as an additional criterion for fatal poisoning with ethanol and drugs (skeletal muscle isoform).

To show that direct electron transfer reactions of heme proteins can be used not only for H₂O₂ or NO-biosensors construction, but also, what is more important, for direct detection of heme proteins in biological liquids, the SPE/CB/DDAB sensor was tested as a model for Mb determination in solutions. Aliquot of Mb buffer samples were placed onto the SPE/CB/DDAB sensors' surfaces. Sensors were allowed to stay for 15 min at 37 °C. Then electrochemical measurements were performed by SWV. The calibration curve of Mb reduction at the surface of SPE/CB/DDAB sensor is shown in Figure 4.

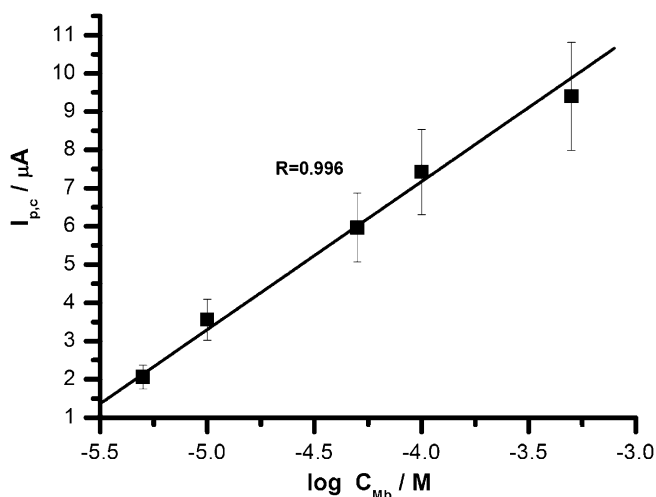


Fig. 4. Calibration curve of Mb detection with SPE/CB/DDAB. Aliquots of Mb solutions were 0.25 μL; PB, pH 6.5. SWV: $E_i = 0.1$ V; $E_c = -0.6$ V; $\Delta E_p = 20$ mV; $\Delta E_s = 5$ mV; $f = 10$ Hz.

The peak current variation ($I_{p,c}$ (μA)) vs. the logarithm of myoglobin concentration ($\log C_{Mb}$ (M)) was linear within the range 5 to 500 μM. Its corresponding regression equation was $I_{p,c}$ (μA) = $23 \pm 3 + 3.9 \pm 0.5 \log C_{Mb}$ (M) ($R = 0.996$ and $n = 5$). Relative standard deviation for three measurements was 15%. The sensitivity of Mb determination with the SPE/CB/DDAB sensor was about an order higher than it was with the CB-unmodified sensor surface (data not shown). Therefore, the direct electron transfer reaction of Mb can be used for qualitative measurement of the heme protein from solution, and

CB can be effectively used as a modifier in electrochemical Mb-biosensors.

3.5 cMb-Immunosensor (SPE/CB/DDAB/anti-cMb): Analysis of Human Blood Plasma Samples

As an electrode surface modifier, CB was applied for cMb-immunosensor construction. For this purpose, cMb-antibodies were immobilized on the CB/DDAB matrix. Plasma samples from healthy donors and patients with acute myocardial infarction were analyzed. Figure 5 pres-

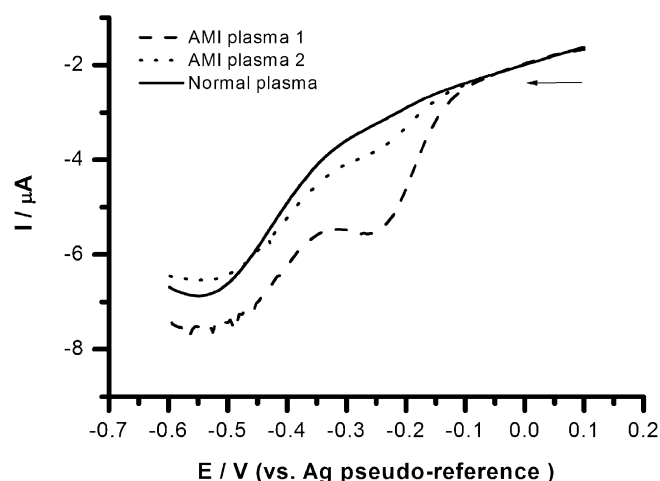


Fig. 5. SWVs of cMb reduction using SPE/CB/DDAB/anti-cMb immunosensors. Plasma samples from healthy donor and patients with myocardial infarction with $C(\text{cMb}) = 350 \pm 35$ ng/mL (AMI plasma 1) and $C(\text{cMb}) = 190 \pm 19$ ng/mL (AMI plasma 2) were analyzed. 0.25-μL aliquots of plasma samples were used. SWVs were registered in PB, pH 6.5 at $E_i = 0.1$ V; $E_c = -0.6$ V; $\Delta E_p = 20$ mV; $\Delta E_s = 5$ mV; $f = 10$ Hz.

ents typical SWVs of Fe(III)-cMb reduction from the plasma sample registered with the SPE/DDAB/anti-cMb and SPE/CB/DDAB/anti-cMb sensors. Following CB-modification a one order increase in the cathodic peak currents was registered for Mb in human blood plasma samples (data not shown), which was in agreement with the data obtained for buffer samples with sensor without antibodies (SPE/CB/DDAB, Section 3.4, Figure 4).

4 Conclusions

Commercially available CB N220 in combination with the surfactant DDAB was found to be a perspective matrix for investigation of the direct electron transfer between heme proteins and electrode. Well-shaped cathodic and anodic peaks were registered by CV and SWV on SPE/CB/DDAB electrodes for cyt *c*, Mb, HRP and CYPs (both in solution and in immobilized state). The direct electrochemistry of heme proteins on CB/DDAB-modified electrodes allowed us to obtain a successfully-oper-

ating immunosensor for cardiac myoglobin detection in human blood plasma.

The advantages of DDAB/CB matrix for heme proteins' direct electron transfer registration were achieved due to a combination of such beneficial factors as electrocatalytic and hydrophilic properties of the liquid crystal surfactant DDAB, its good adsorptive capability, and the unique structural features of CB. As a carbon electrode surface modifier in electroanalytical chemistry, CB can compete with SW CNT, especially with respect to the signal-to-noise ratio, simplicity of preparation of dispersion and low cost (around 1 euro per 1 kg). Potential applications of electrochemical activity exhibited by HRP and CYPs involve the usage of HRP- and CYPs-catalyzed reactions for biosensors construction and for screening potential substrates and inhibitors of these heme proteins. Mb detection is highly necessary for express diagnosis of AMI (cardiac isoform) and other diseases and disease states. Cyt *c* electrochemistry is important for investigation of the respiratory chain and for elucidation of the mechanism of heme proteins' autooxidation (as nonbinding oxygen control).

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