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# Direct electrochemistry of myoglobin immobilized on non-modified and modified graphite

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Immobilization of redox protein myoglobin (Mb) through adsorption was optimized so that a direct redox transformation of the protein active site onto the surface of spectroscopic graphite was observed. The phenomenon of the direct electron transfer (DET) between the myoglobin redox centre and the electrode surface, detected both on non-modified and on graphite, patterned with gold nano-deposits, was studied using a wide range of electrochemical methods such as linear and cyclic voltammetry, square-wave voltammetry, and differential pulse voltammetry. It was shown that the immobilized Mb exhibits a high catalytic activity at the electroreduction of $H_2O_2$.

**Key words**: myoglobin, immobilized, graphite, direct electron transfer

## INTRODUCTION

The direct electron transfer (DET) between immobilized biocatalysts (redox enzymes or proteins) and the electrode surface is a key factor in the development of sensitive and fast responding electroanalytical devices (biosensors), on the one hand, and miniature electrochemical sources of electricity, the so called biofuel cells, on the other hand.

The occurrence of direct electrical communication between the immobilized biocatalyst and the electrode surface, provides a series of advantages in the designed on this basis amperometric biosensors, in terms of the: operating simplicity, cost-efficiency, high sensitivity and selectivity, relatively long linear dynamic range of the signal, and relatively good stability. The studies of DET between the proteins and the nano-modified electrode materials attracted plenty of interest [1–11] mainly because of the opportunity they provide to both, connect the biocomponent with the modifier via specific chemical interactions, and spatially orientate it on the electrode during immobilization. That is why the nanostructured materials that bind biomolecules are currently on high demand for the purposes of bioanalytical chemistry and biotechnologies.

An overview of the current state of the art in the bioelectrochemical area shows that the development of systems based on direct bioelectrocatalysis has been achieved only for a limited number of enzymes, mainly for some heme- and Cu- containing redox enzymes (peroxidases, catalases, laccases, etc.) [12–14]. The great interest towards heme-containing redox proteins myoglobin (Mb) and hemoglobin [1–11, 15–20], is mainly due to their capabilities to perform direct electrical communication with modified electrodes, and hence to the new prospects that this type of heterogeneous bioelectrocatalysis opens up for sensor technologies. What is typical of these two redox-proteins is not only the structural similarity between their active sites but also their tendency to perform direct electron transfer (exchange) with carbonaceous electrodes, such as pyrographite [15–17], carbon paste [18] or glassy carbon [1-2]. In most of these cases myoglobin has been immobilized on electrode materials by including the redox-protein in polymer films: polyethylenimine [15], p-hydroxybutyrate (a naturally occurring lipid polymer) [16], ionomer Eastman AQ38 [17], through the formation of zol-gel [18], in a layer of hexagonal mesoporous silica (HMS) [2], in the non-toxic and possessing good biocompatibility films of histidine [19] and chitosan [20].

The purposeful building of complex electrode architecture by patterning its surface is a novel approach to immobilizing the biocomponent, aimed at achieving direct electrical communication between the biocomponent and the electrode through: 1) shortening the distance between the

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electrode surface and the active centre; and 2) orientating the biocatalyst in a conformation favorable for DET. In this connection, the modification of electrode materials with nanoparticles of $Z_rO_2$ [1], with gold nanoparticles [3–6], carbon nanotubes (CNT) [8–10], and titanium nanotubes (TiNT) [11], prior to immobilizing myoglobin in all cases has resulted in direct bioelectrocatalysis. The capacity and possibilities of these bioelectrocatalytic systems are determined by the unique properties of the nanoparticles. They are characterized by a high surface-to-volume ratio and provide a reliable and compact surface for the oriented adsorption of biomolecules while retaining their biological activity. In most cases, the very Au-nanoparticles represent a kind of nano-wires, facilitating the electron transfer between the electrode and the protein. The employed modification approaches allow for the size and the surface morphology to be controlled through varying the conditions for depositing them. In all investigated cases, the direct bioelectrocatalysis with immobilized myoglobin offers a good basis for the development of extremely selective biosensors for qualitative determination of $H_2O_2$, $O_2$, $CCl_3COOH$ and $NaNO_2$.

During the past decade two research streams became traditional for our group: a) studies on the electrocatalytic activity of compact carbonaceous materials, modified with biological catalysts, redox-proteins and enzymes from the group of the oxidoreductases; and b) development and characterization of electrocatalysts through electrochemical deposition of micro- and nanostructures of precious metals (Pd, Pt, Au, Ir, etc.). This study brings together to a great extent the above specified two research directions of the group, and its objective is to investigate the direct electrochemistry of myoglobin (Mb), immobilized on both, non-modified and graphite, modified with gold nanodeposits, as well as to examine its electrocatalytic activity at the reduction of $H_2O_2$

## EXPERIMENTAL

### *Materials*

Myoglobin, purified from horse heart muscle, was used in this research work (myoglobin from horse heart: Fluka Biochemika).

$H_2O_2$ and the chemicals for preparing the buffer solutions ($Na_2HPO_4.12H_2O$, citric acid, conc. $H_3PO_4$ and Tris (hydroxymetyl – aminomettane), were all purchased from Fluka. Gelatin for analytical application, manufactured by Chimtek Bulgaria, was used for the film coating of the modified electrodes as a gel (5% in phosphate-citrate buffer, pH 7.0). All solutions were prepared with double-distilled water.

The working electrode was made of spectroscopically pure graphite, in the shape of a disc with a diameter of 0.5 – 0.6 cm, pressed (extruded) together with Teflon, with a copper current lead.

### *Preparation of the electrodes:*

a/ the gold nanoparticles were deposited onto graphite electrode through the following procedure: mechanically cleaned and rinsed with double distilled water electrode was used as working electrode in a conventional three-electrode cell (with non-separated compartments), filled with a 2% solution of $HAuCl_4$ + 0.1M HCl, followed by a brief electrolysis (t = 10 s) at $E_r^{dep} = +0.05V$, *vs.* a reversible hydrogen electrode.

b/ both on non-modified and modified with gold nanodeposits graphite, redox protein Mb was adsorbed under static conditions from a solution containing 2 $mg \cdot ml^{-1}$ Mb, dissolved in Tris-buffer, pH = 7. The duration of the adsorption process was from 30 to 60 min., and was followed by electrode air-drying. In addition, a drop of 20 μl of the same Mb solution was deposited on the electrode surface and left to dry in air.

### *Equipment and measurements*

All electrochemical measurements were performed in a non-compartmentalized three-electrode cell with a working volume of 10–15 ml. Ag/AgCl (1M KCl) was used as a reference electrode, and a platinum wire – as an auxiliary electrode.

Cyclic, square-wave and differential pulse voltammetry were performed using Palm Sens electrochemical workstation ( Palm Sens BV, the Nederland).

## RESULTS AND DISCUSSION

Fig.1 shows cyclic voltammetric curves (CVs) of the myoglobin, adsorbed on bare graphite in a background electrolyte phosphate-citrate buffer, pH = 7. The voltammograms were registered at different scan rates ranging from 1 to 40 $mV \cdot s^{-1}$. Fig. 1 shows diffuse maxima on the anode (over the region from – 0.05 to 0.10 V) and the cathode (at the reverse scan from –0.30 to – 0.10 V) parts of the voltammogram. Peak reversibility was established only at the lowest rate

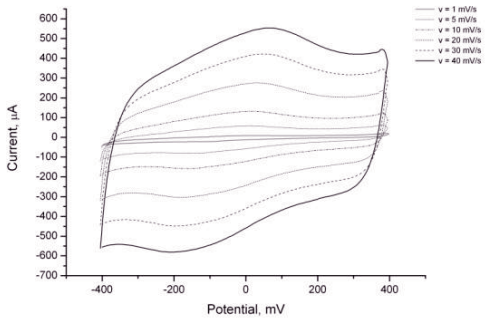


**Fig.1.** Cyclic voltammograms of adsorbed on graphite myoglobin; phosphate – citrate buffer pH 7.0 ; scan rate of 1, 5, 10, 20, 30, 40 $mV \cdot s^{-1}$ (from inner to outer) ; reference electrode Ag/AgCl, 1M KCl.

of 1 $mV \cdot s^{-1}$. The peaks are reversible at this rate since the following criteria applied for both maxima:

$$I_a^* / I_k^* = 1 \qquad \text{and}$$

$$\Delta E = E_a^* - E_k^* = \Delta E^* = \frac{2{,}303RT}{nF},$$

where $I_a^*$ and $E_a^*$ are the values of the current and the potential of the anode peak, respectively; and $I_k^*$ and $E_k^*$ are the analogous values of the cathode peak.

The criterion that apply at scan rates between 5 and 20 $mV \cdot s^{-1}$ is :

$$\Delta E = E_a^* - E_k^* = \frac{2{,}303RT}{\alpha nF},$$

which suggests a quasi-reversible electrochemistry of the adsorbed myoglobin. At scan rates higher than 20 $mV \cdot s^{-1}$, the process turns practically irreversible, as peak separation $\Delta E^* > 200$ mV. Upon increasing scan rate, the anode maxima shift in a positive direction whiles the cathode maxima move in a negative direction. At v = 40 $mV \cdot s^{-1}$, the anode maximum was found to be at a potential of

$$E_a^* = 60 \pm 5mV,$$

and the cathode's at

$$E_k^* = -180 \pm 10mV.$$

Such redox maxima were not observed on the CVs of the bare graphite electrode (without adsorbed Mb, not shown in Fig.1). Therefore, their occurrence is due to the redox transformation of the myoglobin, immobilized on graphite. This transformation involves the iron in the heme of its

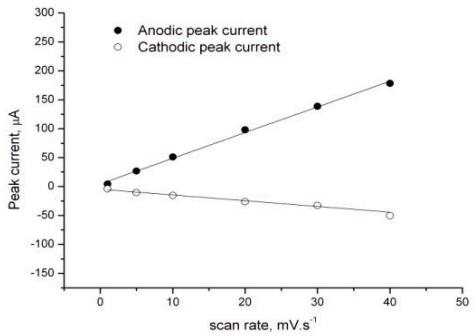


**Fig.2.** Peak current *vs.* scan rate of anodic (upper graph curve) and cathodic (bottom curve) peak current ($\mu A$) of myoglobin adsorbed on graphite.

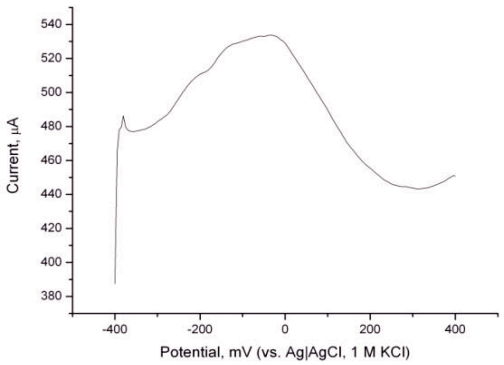


**Fig.3**. Square wave voltammetry of adsorbed on graphite myoglobin; phosphate- citrate buffer pH 7.0; reference electrodeAg /AgCl, 1M KCl.

active centre. The number of electrons, taking part in the redox process, has been calculated on the basis of the equation

$$\Delta E^* = \frac{2{,}303RT}{\alpha nF},$$

assuming the transfer factor

$\alpha = 0.5$. At $\Delta E^* = 100$ mV,

the number of electrons is n = 1.16, i.e. one electron exchanged during the redox process.

The dependence of the anode current (upper curve) and the cathode (lower curve) maxima on the scan rate is depicted in Fig.2. The linear trend of this dependence shows that the process, which takes place on the graphite, is controlled by surface chemistry, i.e. it takes place in a thin surface layer.

The electrochemistry of the myoglobin, immobilized on non-modified graphite, was also examined by means of the square-wave voltammetry (Fig.3). The formal redox potential of

the heme in the myoglobin active site was determined to be:

$$E_{red./ox.} = \frac{E_a^* + E_k^*}{2} = -30 \pm 20\ mV$$

(due to the broad maximum observed in Fig.3).

The potential, determined through the SWV method, $E_{red/ox}$, is completely consistent with the data presented, which were obtained by cyclic voltammetry (see Fig.1).

Fig.4 shows the cyclic voltammogram of myoglobin, adsorbed on graphite modified with gold nanoparticles. Reversible anode and cathode maxima can be observed on the CV, the region from –450 mV to –250 mV (on the anode curve) and from –500 mV to –250 mV (on the cathode curve). Their reversibility was examined according to the two criteria, specified above. The values of the currents $I_a^*$ and $I_k^*$ in applying the criteria, are determined as shown in Fig.4, subtracting the background current. This finding evidences the complete reversibility of the electrochemical reaction which takes place on the modified graphite electrode with immobilized myoglobin.

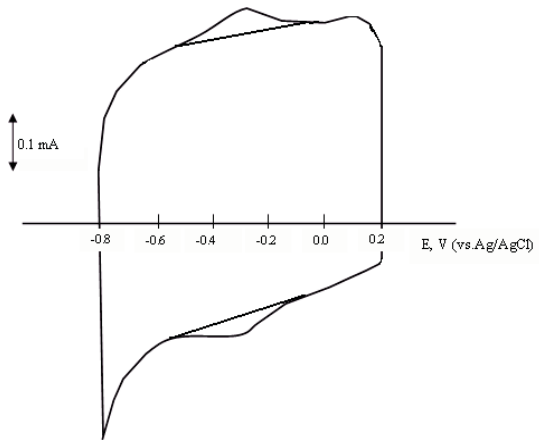


**Fig. 4**. Cyclic voltammogram of myoglobin, adsorbed on modified whit Au-nanodeposits graphite; phosphate-citrate buffer pH 7.0; scan rate 80 mV·s$^{-1}$; reference electrode Ag/AgCl.

The fact that it is possible to register reproducible I, E curves continuously, proves that myoglobin is adsorbed irreversibly on graphite, modified with gold nanoparticles. In addition to the high biocompatibility of the gold nanoparticles, the facilitated electron transfer between the protein redox site and the electrode surface, is also due to the specific binding of thiolated terminal groups from the protein shell of the myoglobin with gold, i.e. this is the so called self-assembly of the protein onto the gold surface, which is a typical chemosorption process.

The atomic-force microscopy of the graphite, modified with gold nanoparticles, revealed (Fig.5) that the gold nano-wires, formed during the modification, reached a height of up to 100 nm, thus also contributing to shortening the distance between the myoglobin active site and the modified surface.

The voltammetric studies of the graphite, modified with immobilized myoglobin (Fig.4), shows that oxidation and reduction of the protein take place at a potential of

$$E = -300 \pm 20 \text{ mV}.$$

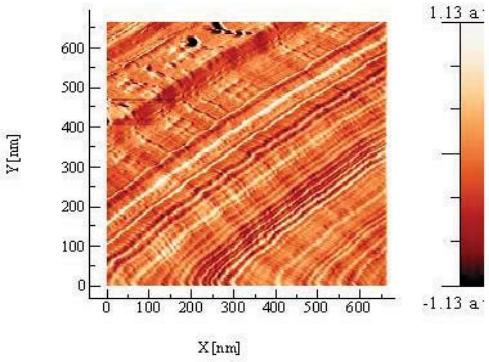


**Fig. 5a**

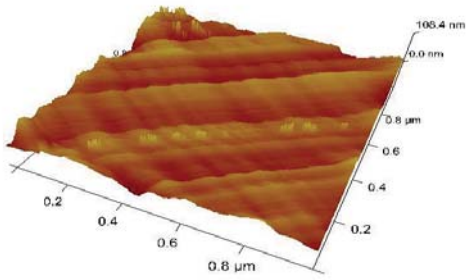


**Fig. 5b**

**Fig .5**. 3D- topology of modified with Au-nanodeposits graphite studied whit AFM.

The number of electrons, calculated from the correlation of

$$\Delta E^* = 2{,}303\frac{RT}{nF},$$

is also one. Therefore, the redox transformation of the myoglobin, adsorbed on modified graphite, also affects the iron in the heme of the protein active centre, and proceeds with the transfer of one electron. The voltammetric curves of the

myoglobin, adsorbed on modified graphite, are characterized by sharper and more clearly defined maxima (Fig.4) as compared to the curves of the myoglobin, adsorbed on bare graphite (Fig.1). Similarly to the electrochemical behavior of the protein, adsorbed on bare graphite, the cathode peaks, observed on modified graphite, also shift negatively with increasing scan rate (Fig.6).

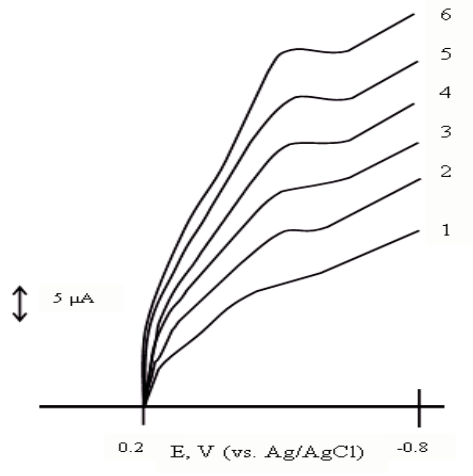


**Fig.6**. Cathodic linear sweep voltammograms of myoglobin, adsorbed on modified with Au-nanodeposits graphite; phosphate-citrate buffer pH 7.0; scan rate: 10-60 $mV \cdot s^{-1}$ (1-6); reference electrode Ag/AgCl, 1M KCl.

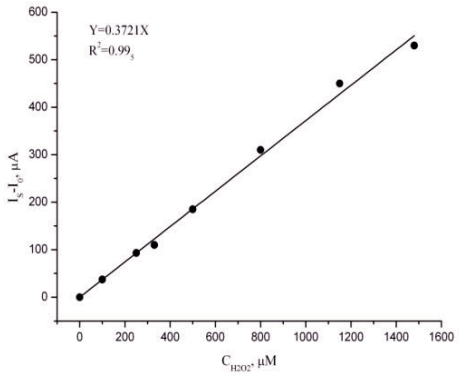


**Fig.7**. The linear regression of the reduction peak current as dependent on increased $H_2O_2$ concentration.

The catalytic activity of myoglobin, adsorbed on graphite, modified with gold nanoparticles, was also examined at the electrochemical reduction of $H_2O_2$ (Fig.7). It can be seen on this Figure that the dependence of the cathodic current on the concentration of $H_2O_2$ is linear over one and a half orders of magnitude, ranging from $1\times10^{-4}$ M to $15\times10^{-4}$ M. The linear regression was shown to obey the equation of y = bx with a correlation factor of $0.99_5$; the sensitivity being dI/dC= 0.372 $\mu A \cdot \mu M^{-1}$. This reaction constitutes a possibility for creating an amperometric biosensor for $H_2O_2$.

## CONCLUSIONS

- An optimal method for adsorption of myoglobin on both, non-modified graphite and on graphite, modified with gold nanoparticles, was found where a direct redox transformation of the protein was observed.
- The voltammetric studies show that depending on the scan rate, the electrochemical reaction of hemoglobin, adsorbed on non-modified graphite, changes from reversible through quasi-reversible to irreversible as the rate increases. This reaction is also not completely reversible on modified graphite.
- The redox transformation of the immobilized myoglobin, both, on non-modified and on modified graphite, involves the iron in the heme of the protein active centre. The redox potential of myoglobin, adsorbed on non-modified graphite, was found to be E =30 ± 20 mV, and on modified graphite - E = –300 ± 20 mV.
- Myoglobin, adsorbed on graphite, modified with Au-nanoparticles, exhibits high electrocatalytic activity in the reduction of $H_2O_2$. This reaction provides a possibility for creating an amperometric biosensor for detection of $H_2O_2$.

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## ДИРЕКТНИ ЕЛЕКТРОХИМИЧНИ ОТНАСЯНИЯ НА МИОГЛОБИН, ИМОБИЛИЗИРАН ВЪРХУ НЕМОДИФИЦИРАН И МОДИФИЦИРАН ГРАФИТ

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(Резюме)

Адсорбционната имобилизация на редокс-протеина миоглобин (Mb) върху повърхността на спектрално-чист графит беше оптимизирана така, че да се осъществи директна електрохимична трансформация на активния му център върхуповърхността на така получения ензимен електрод. Осъществяване на явлението директен електронен пренос (DET) между редокс-активния център на миоглобина и електродната повърхност беше наблюдавано както върху немодифицирани, така и върху модифицирани със златни нано-отложения графити и беше изследвано с разнообразни електрохимични методи: линейна и циклична волтамперометрия, волтамперометрия с квадратен импулс и диференциална импулсна волтамперометрия. Беше показано, че имобилизираният миоглобин притежава висока каталитична активност при електрохимичната редукция на $H_2O_2$.