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Wiring of heme enzymes by methylene-blue labeled dendrimers

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Abstract.

Redox-modified branched 3D dendrimeric nanostructures may be considered as perspective wires for electrical connection between redox enzymes and electrodes. Here, we studied electron transfer (ET) reactions and bioelectrocatalysis of heme-containing horseradish peroxidase (HRP) and heme- and molibdopterin-containing sulfite oxidase (SOx), wired to gold by the methylene blue (MB)-labeled polyamidoamine (PAMAM) dendrimers. The enzymes' electrochemical transformation and bioelectrocatalytic function could be followed at both unlabeled and MB-labeled dendrimer-modified electrodes with the formal redox potentials of the heme centers being at -341 mV for HRP and -185 mV for SOx. In contrast to the HRP-dendrimer and HRP-MB-dendrimer systems, which demonstrated very close bioelectrocatalytic patterns, multifactor SOx wired to MB-dendrimer showed a bioelectrocatalysis superior to that based on direct ET reaction. This can be correlated with a statistically larger number of positively charged MB sites on the dendrimer surface able to electrically connect the negatively charged heme domain of SOx and the electrode. In this perspective, redox-labeled dendrimers appear to be useful tools for wiring, optimization of bioelectrocatalysis of complex intermembrane and, possibly, membrane enzymes.

Key words. Bioelectrocatalysis, Dendrimer, Methylene blue, Horseradish peroxidase (HRP), Sulfite oxidase (SOx)

1. Introduction

Electrochemistry of redox active proteins and enzymes offers a powerful tool for studying the electron transfer (ET) properties of these biomolecules, including mechanisms of ET reactions and their biotechnological applications in biosensors, biofuel cells and bioelectronic devices [1-4]. Heme-containing enzymes whose redox activity relies on the heme, occupy one of the central places in this research and include: 1) complex cofactor enzymes, such as flavo- and heme containing cellobiose dehydrogenase, theophylline oxidase or flavohemoglobins, which redox activity at the electrode can be followed from the potentials of their heme and operate as an ET relay between the electrode and the substrate-converting center [5-7], and 2) one cofactor enzymes, such as peroxidases, which bioelectrocatalytic activity can be followed at potentials higher than those of the heme itself [8]. In the latter, that enables discrimination between the inherent electrocatalytic activity of hemin (e.g. in denatured enzymes) and the intact heme-enzyme redox function [9].

Bioelectrocatalysis of H_2O_2 reduction by most thoroughly studied horseradish peroxidase (HRP) coupled to electrodes in different ways has been observed starting from 0.6 - 0.7 V vs. Ag/AgCl, and correlates with the potentials of peroxidase Compound I and Compound II formation [8, 10]. Oriented immobilization of wild-type recombinant genetically-engineered HRP (rHRP) *via* the His and Cys tags introduced in different positions of the enzyme surface resulted in ET rates between HRP's heme and electrodes exceeding 100 s^{-1} , and, as a result, in the particularly efficient direct ET-based bioelectrocatalytic reduction of H_2O_2 [11]. Along with that, the absolute amount of the electronically wired (and thus bioelectrocatalytically active) peroxidase molecules was estimated to be at a sub-picomolar level, representing less than 1% of the theoretical monolayer coverage [11-13]. In this context, controlled enzyme immobilization resulting in a higher loading of the electrode with the electrically wired and electrocatalytically active enzyme is of evident fundamental and biotechnological interest.

Electrical wiring of the enzyme active sites by proper mediators, such as conductive polymers [14], osmium complex-conjugated polymers [15-17], and electrode-tethered redox relays [18, 19] represent a challenging reagentless alternative to direct ET methodology (when the enzyme undergoes direct electrochemical redox transformation on the electrode surface), though with a higher level of the system complexity. The reagentless mediator approach is universal (*i.e.* applicable to any enzyme) and demonstrated its high value with such biotechnologically important enzyme as glucose oxidase, used for construction of glucose sensors for diabetes monitoring [20]. From the strategic point of

view, synthetic redox relays are of a special biotechnological value since they allow a controlled design and self-assembly of electrically wired multi-enzyme complexes onto electrodes. Such electronic inter-communication can provide the artificially designed ET chains and metabolic enzymatic cascades with the required properties for exploitation in nanobiotechnology [4, 21].

Synthetic redox-modified branched 3D dendrimer nanostructures with their controllable topology, precise position, variety of redox and catalytic labels that can be used for their modification, and straightforward labelling and handling protocols [22] may be considered perfectly suited for such bioelectronic applications. In particular, ferrocene- (Fc) and ruthenium complex-terminated synthetic poly(amido)amine dendrimers were shown to be robust electron transferring supramolecular blocks [23-25] with all terminal redox groups involved in the ET reaction. There were also several attempts to wire some enzymes, such as glucose oxidase, by Fc-labeled dendrimers, though, it was not clear if the Fc-labeled dendrimer worked as a wire or just the Fc groups adjacent to the electrode surface [26, 27].

Figure 1. Schematic representation of the gold electrode surface modification with PAMAM dendrimers, their functionalization with methylene blue (MB) and enzyme wiring.

In our previous work we studied in detail the ET properties of methylene-blue (MB) labeled dendrimers tethered to gold, which exhibited ET rates approaching 267 s^{-1} [28], much faster than those on a slower electrode material, graphite, with only 7 s^{-1} reached [29]. Therewith, a very special electrochemical behavior of the MB-dendrimers was demonstrated: the mechanism of ET reaction in redox-labeled dendrimers switched between electron tunneling and electron hopping. This was a function of the dendrimer surface coverage and the potential scan rate (in other words, the timescale of the experiment), both inducing dendrimer structural changes on the electrode surface [28]. Those results were consistent with the earlier discussed electron hopping vs. electron tunneling reactions in the Ru-complex-labeled dendrimers [23].

The impressive ET properties of the gold-tethered MB-dendrimers were expected to provide an efficient enzyme wiring to the electrodes, possibly, faster than the enzyme itself can undergo. Along with that, the efficiency of the wire might be compromised by complex mechanisms of electron transport in the dendrimer systems. In the current work, we have studied the ET reactions and bioelectrocatalysis of heme peroxidase, HRP, and complex heme- and molibdopterin-containing sulfite oxidase (SOx) wired to gold by the MB-dendrimers (Figure 1).

2. Experimental

2.1. Materials.

Components of buffer solutions, di(N-succinimidyl)-3,3'-dithiodipropionate (DTSP), dimethyl sulfoxide (DMSO), hydrogen peroxide solution (30 % w/w), sodium sulfite (98%), methylene blue, G3-poly(amidoamine) dendrimer (PAMAM) and methanol of analytical grade or of ultra-high purity were purchased from Sigma-Aldrich (Germany) and used as received. Native horseradish peroxidase (nHRP, 330 U mg^{-1}) was from Sigma-Aldrich; the recombinant form of wild type HRP expressed in *E. coli* (rHRP, 600 U mg^{-1}) was kindly provided by Prof. Irina Gazaryan (Burke Medical Research Institute, New York City, USA)[11]. A suspension of 7.8 mg mL^{-1} sulfite oxidase (SOx, 24.3 U mg^{-1}) from chicken liver in 3.2 M ammonium sulfate containing 1.6 mM molybdic acid (pH 7.5) was purchased from Sigma-Aldrich (Denmark). N-hydroxysuccinimide ester (NHS)-activated monocarboxymethylene blue (MCMB-SE) was acquired from empBiotech (Berlin, Germany). De-ionised Milli-Q reference A^+ water purification system ($18\text{ M}\Omega$, Millipore, Bedford, MA, USA) was used all over the work for preparation of solutions.

2.2. Preparation of the dendrimer-modified electrodes.

Gold disk electrodes (0.2 cm diameter) were electrochemically cleaned in 0.5 M NaOH by cycling at 50 mVs⁻¹. Afterwards they were hand-polished to a mirror luster with 1 µm diamond and 0.1 µm alumina slurries (Struers, Denmark) on microcloth pads (Buehler, Germany) and sonicated in EtOH/water (1:1) for 30 min. The polished gold electrodes were dried under nitrogen stream. After mechanical polishing the electrodes were electrochemically pretreated by cycling in 1 M H₂SO₄ and 0.5 M H₂SO₄/10 mM KCl at 300 mVs⁻¹. The electroactive surface area was determined by integrating the charge under the reduction peak of gold surface oxide during the final scanning in 0.1 M H₂SO₄ and assuming a theoretical value of 400 µC cm⁻² for a monolayer of chemisorbed oxygen on gold electrode [30]. The electrochemical surface area of the bare gold electrode was typically 0.089±0.003 cm². Clean electrodes were kept in EtOH for 30 min prior modification.

Gold-coated PAMAM films were prepared following the previously published protocol [28]. Briefly, clean gold electrodes were incubated in a 4 mM DTSP solution in dimethyl sulfoxide for 1 h at rt. The produced N-succinimidyl-3-thiopropionate (NSTP)-modified Au electrodes (referred further as NSTP/Au electrodes) were allowed to immediately react with either 0.2 or 18 mM PAMAM solutions in ethanol for 2 h at rt. Succinimide ester-modified MB was then covalently coupled to the PAMAM/NSTP/Au electrodes by incubating the modified electrode in the 2 mg mL⁻¹ MB solution overnight at 4 °C. Subsequently the MB/PAMAM/NSTP/Au electrodes were gently washed with MQ water, dried, and then stored in the buffer solution at 4 °C when not in use.

2.3. Enzyme immobilization on modified electrodes

Immobilization of nHRP, rHRP and SOx on PAMAM dendrimer modified electrodes was performed by placing a 10 µL drop of either 1 mg mL⁻¹ HRP solution in 50 mM phosphate buffer solution, pH 7 (PBS) or 36 µM SOx solution in 0.1 M Tris-HCl, pH 7.4. The electrodes were incubated with the enzyme at rt for 1 h. The enzyme-modified electrodes were carefully rinsed with the corresponding buffer solution and stored in it at 4 °C when not in use.

2.4. Instrumentation.

Cyclic voltammetry (CV) was performed in a three-electrode cell, with a gold disk working electrode (CH Instruments, Austin, TX), Ag/AgCl (3 M KCl) as a reference electrode (Metrohm, Denmark), and platinum wire as a counter electrode. Measurements were carried out with a potentiostat AUTOLAB PGSTAT 30 (Eco Chemie B.V., Utrecht, the Netherlands) equipped with NOVA 1.10

software. Working solutions were de-aerated with N₂ for at least 10 min prior data recording and kept under N₂ during the entire experiment. All experiments were carried out at 22±1 °C.

3. Results and Discussion

Electrochemistry and bioelectrocatalysis of HRP and SO_x both on unlabeled- and MB-labeled dendrimer-modified electrodes have been studied and their relative efficiency of ET reactions and bioelectrocatalysis have been compared.

3.1. ET reactions of rHRP at PAMAM/NSTP/Au modified electrodes

No significant electrochemical response was obtained with native glycosylated nHRP, and all further experiments were performed with de-glycosylated recombinant wild-type form, rHRP [31]. It was shown that due to the absence of carbohydrate residues non-glycosylated rHRP tends to adsorb on gold more efficiently [32]. This provides better direct ET communication between the electrode and the heme group whose Fe³⁺/Fe²⁺ electrochemistry can be followed by DPV analysis [11]. However, the amount of electrochemically wired rHRP molecules adsorbed on gold is so small that it rather allows determination of the formal redox potentials of rHRP's heme (at - 0.422 V) than the amount of wired enzyme [11].

A pronounced electrochemical response from Fe³⁺/Fe²⁺ redox couple of HRP's heme was observed when rHRP was electrostatically adsorbed on PAMAM layers prepared by deposition from 0.2 mM and 18 mM PAMAM solutions (Figure 2), which leads to diluted and saturated PAMAM monolayers respectively, with a characteristic surface coverage (Γ_{PAMAM}) [28]. Consistent with the previously reported data on immobilization of such heme enzymes as myoglobin, hemoglobin and HRP [33, 34], the PAMAM films are expected to provide a favorable nano-environment for rHRP immobilization and electrical wiring. The formal potential (E^0) of rHRP on PAMAM dendrimer films, estimated as the midpoint of reduction and oxidation peak potentials, was - 341±7 mV, which is 81 mV more positive than the potential of the HRP's heme Fe³⁺/Fe²⁺ redox couple on bare gold and the values determined by redox titration in solution [35, 36]. This can be ascribed to rHRP coupling to the hydrophobic and positively charged dendrimer layer and may reflect interactions that stabilize the Fe (II) state of the enzyme.

Figure 2. Representative cyclic voltammograms of rHRP electrostatically adsorbed on **A)** 0.2 mM PAMAM/NSTP/Au and **B)** 18 mM PAMAM/NSTP/Au in the **(1)** absence and **(2)** presence of 0.1 mM H₂O₂ in N₂-saturated 50 mM PBS (pH 7) at a scan rate of 5 mVs⁻¹. Insets: CVs recorded for rHRP/MB/PAMAM/NSTP/Au at scan rates of 10, 25, 50, 70, 90, 100, 125, 150, 200 mVs⁻¹ and dependence of the CV peak current on the potential scan rate (v). $\Gamma_{\text{rHRP}/0.2\text{mM PAMAM/NSTP/Au}} = 17 \pm 2$ pmol cm⁻² and $\Gamma_{\text{rHRP}/18\text{mM PAMAM/NSTP/Au}} = 14 \pm 2$ pmol cm⁻². CVs were recorded at < 0.2 V to avoid oxidative desorption of thiols.

The voltammetric peaks in CVs recorded with rHRP adsorbed on diluted ($\Gamma_{\text{PAMAM}} = 0.8$ pmolcm⁻²) [28] and saturated ($\Gamma_{\text{PAMAM}} = 9.6$ pmolcm⁻²) [28] PAMAM dendrimer layers were used to determine the surface amount of electrically wired rHRP (Γ_{rHRP}). Their integration yield the charge (Q) consumed in the ET reaction according to the equation $\Gamma_{\text{rHRP}} = Q/nFA$ [37], where n is the number of electrons involved in the process, F is the Faraday constant and A is the electroactive surface area of the electrode. Both dendrimer layers showed a comparable and quite close amount of rHRP adsorbed and electrically wired ($\Gamma_{\text{rHRP}/0.2\text{mM PAMAM/NSTP/Au}} = 17 \pm 2$ pmol cm⁻² and $\Gamma_{\text{rHRP}/18\text{mM PAMAM/NSTP/Au}} = 14 \pm 2$ pmol cm⁻²). This suggests that the enzyme surface concentration and further bioelectrocatalytic activity is not essentially affected by the dendrimer surface concentration, but depends on the number of enzyme molecules in close proximity to the electrode surface with a proper orientation for direct ET reaction. Both surface concentration values constitute ca. 67% from the total surface coverage of rHRP on gold (30 pmol cm⁻²) determined by the quartz crystal microbalance [32]. However, they are considerably higher than the amount of electrically wired rHRP directly adsorbed on gold (0.17 ± 0.03 pmol cm⁻² [11]) or attached to gold through diazonium salt grafting (3.3 pmol cm⁻² [38]). Thus, the dendrimer-modified electrodes demonstrated their high loading capability with electrically wired enzyme, comparable to other carbon nanostructures, such as carbon nanotubes (24 pmol cm⁻² [39]) (Table 1).

As expected, both oxidation and reduction peak currents in CVs (Figure 2, insets) increased linearly with the scan rate in the range of 0.01 to 0.2 Vs⁻¹ designating a diffusionless, surface-confined process

[40]. The apparent heterogeneous ET rate constant (k_s) was estimated according to Laviron, from the peak-to-peak separation ΔE ($\Delta E < 200/n$ mV) by using the tabulated values of the k_s -related parameter m [37]. For rHRP on the diluted PAMAM monolayer, the k_s was 2.8 ± 0.2 s⁻¹, almost the same as 2.9 ± 0.4 s⁻¹ obtained for the saturated PAMAM monolayer. These are comparable with the k_s values shown for nHRP on gold (3.7 ± 0.3 s⁻¹) [41], graphite (2.5 ± 0.6 s⁻¹) [42], or single-walled carbon nanotubes/glassy carbon (4.7 ± 0.3 s⁻¹) [39] electrodes, but are 10-fold lower than the rates of direct ET between the arbitrarily adsorbed rHRP and gold (28 ± 4 s⁻¹) [11] (Table 1). Thus, the gold-coated PAMAM dendrimer films may be considered as a kind of enzyme-friendly cushion that saves the enzyme integrity at the electrode, but also contributes to slower the rates of ET reaction, probably due to the effect of positively charged PAMAM on rHRP orientation at gold, affecting the ET pathways.

Table 1. The surface coverage, Γ_{HRP} , and the heterogeneous ET rate constant, k_s , between the heme of HRP and the electrodes determined for differently HRP-modified electrodes

Electrode modification	Γ_{HRP}		k_s		Ref.
	Method	pmol cm ⁻²	Method	s ⁻¹ (pH 7)	
rHRP/PAMAM/NSTP/Au	CV *	Electrically wired: 17 ± 2	CV	2.8 ± 0.2	This work
rHRP/Au	QCM	Total amount: 30	—	—	[32]
rHRP/Au	DPV	Electrically wired: 0.17	CA, RDE	28 ± 4 s ⁻¹	[11] Figure 4
native HRP/Au	QCM data from [32]	Total amount: 23	CA, RDE	3.7 ± 0.3	[41]
native HRP/diazonium functionalized Au	CV *	Electrically wired: 3.3	CV	1	[38]
native HRP/viologen SAM/Au	CV *	Electrically wired: 65	CV	3.41	[43]
native HRP/PTMSPA@GNRs	CV *	Electrically wired: 45	CV	3.2 ± 0.1	[44]
native HRP/Graphite	Approximation to a monolayer, assumed	Total amount: 40	CA, wall-jet FIA	2.5 ± 0.6	[42]
native HRP/Graphite	Theoretical fitting to kinetic analysis data	Electrically wired: 0.15	LSV, RDE	1	[13]
native HRP/TCAP/GCE	Spectrometric	Apparent amount: 1.2	CV	1.03	[45]
native HRP/SWCNT/GCE	CV *	Electrically wired: 24	CV	4.7 ± 0.3	[39]

PTMSPA: poly(N-[3-(trimethoxysilyl)propyl]aniline); GNRs: Gold nanorods; TCAP: 5,2':5',2''-terthiophene-3'-carboxylic acid polymer; GCE: Glassy carbon electrode; SWCNT: single-walled carbon nanotubes; CV: cyclic voltammetry; QCM: quartz crystal microbalance; DPV: differential pulse voltammetry; CA: chronoamperometry; RDE: rotating disk electrode; FIA: flow injection analysis; LSV: linear sweep voltammetry; *Integration of the Fe³⁺/Fe²⁺ heme peaks

In the presence of H_2O_2 two reactions proceed in the system (Figure 2). The first occurs at potentials more positive than HRP's $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple (from 0.2 to -0.1 V). This is related to the bioelectrocatalytic reduction of H_2O_2 by the HRP's heme with the formation of its higher oxidation state known as Compound 1 ($E1$) [8, 11], represented by an oxyferryl iron ($\text{Fe}^{4+}=\text{O}$) and a porphyrin π cation radical ($\text{P}^{\bullet+}$). The reduction of $E1$ by a two-electron donor leads to an intermediate Compound II ($E2$) and returns the enzyme to its original ferric state $\text{HRP}(\text{Fe}^{3+})$ (reactions 1 and 1'). The formal potentials of $E1$ and $E2$ were reported as $E_1^{0'} = 0.72$ V and $E_2^{0'} = 0.75$ V vs. AgCl/Ag electrode at pH 7 [10]. The second bioelectrocatalytic process proceeds at potentials of HRP's $\text{Fe}^{2+}/\text{Fe}^{3+}$ heme couple, with a significant increase in the $\text{HRP}(\text{Fe}^{3+})$ reduction peak and the disappearance of the oxidation peak from the reduced heme. This is consistent with the bioelectrocatalytic reduction of H_2O_2 by the $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple of HRP (reactions 2 and 2').

Based on the observed currents, the apparent bioelectrocatalytic efficiency of H_2O_2 reduction by reaction route (1) and reaction route (2) was very different. The estimated from the ratio between the $\text{Fe}^{3+}/\text{Fe}^{2+}$ catalytic and non-catalytic peak currents was around 10 (10.4 and 9.7 for diluted and saturated films, respectively), while for the $E1$ was 4.2 if the currents are taken at potentials more positive than HRP's $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple (*i.e.* at 0 V). It is important to note that when the efficiency of the process (1, 1') is high, the direct ET signals from the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple of HRP's heme are almost negligible [11]. However, in the case of a pronounced $\text{Fe}^{3+}/\text{Fe}^{2+}$ electrochemistry (excluding the works very likely reporting data for denatured enzymes) the most efficient bioelectrocatalysis starts from the potentials of the heme $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox couple [46]. It remains the question, why despite the pronounced $\text{Fe}^{3+}/\text{Fe}^{2+}$ redox chemistry of rHRP, significantly lower bioelectrocatalytic currents from H_2O_2 reduction are observed at higher potentials (in some other works, those are not even detected). Technically, such differences in bioelectrocatalytic efficiencies of processes (1) and (2) may be connected with either different number of rHRP molecules involved in reactions 1 and 2 or different values of the k_s and k_s^* . The former depends on the proper orientation

of the enzyme for ET between the electrode and the enzyme redox group, while the latter involves conformational changes in the active site of the enzyme, which are expected to be more pronounced in the case of reaction 1.

3.2. ET reactions of rHRP at MB/PAMAM/NSTP/Au modified electrodes

To improve the rate of ET between the enzyme's redox center and the electrode surface the PAMAM dendrimer films were modified with the MB redox probe, which at pH 7 exhibits a $2e^-/1H^+$ ET reaction [28, 29]. MB was employed as an electron-transfer mediator that did not exhibit any catalytic activity towards H_2O_2 reduction (Figure S1). The MB-dendrimer film showed a couple of redox peaks centered at -189 ± 1 mV (Figure S2) consistent with previous reports [28]. This formal potential is about 35 mV more positive than the reported for MB in solution, and conclusively results from the MB attachment to a quite hydrophobic and positively charged dendrimer layer [47]. A similar shift of the MB redox potential was also observed for MB-dendrimer films on graphite [29]. It is also worthwhile to note the high peak symmetry of MB (Figure 3, curve 1) and the small peak separation at essentially low scan rates ($\Delta E = E_{pa} - E_{pc} = 13 \pm 4$ mV, at 100 mVs^{-1}), characteristic/approaching the “reversible peaks” case. The peaks have a full-width-at-half-maximum (fwhm) of 82 ± 11 mV, almost double than the theoretical value of 45 mV for a $2e^-$ Nernstian process. This supports a $1e^-$ transfer reaction, seemingly to be the case when MB is bound to supramolecular structures such as nucleic acids [48-51] or dendrimers [28, 29]. Our previous experiments have shown that the ET properties of MB-dendrimer films are strongly influenced by the amount of dendrimer molecules on the surface. Diluted films ($\Gamma_{\text{MB/PAMAM/NSTP/Au}} = 24 \pm 1 \text{ pmol cm}^{-2}$) acted as electrical wires with ET rates approaching $267 \pm 1 \text{ s}^{-1}$, while in the saturated ones ($\Gamma_{\text{MB/PAMAM/NSTP/Au}} = 267 \pm 7 \text{ pmol cm}^{-2}$) the restricted conformational mobility of the dendrimer branches impeded the necessary deformation of dendrimers in the electric field for efficient ET, decreasing the rate of ET to $26 \pm 9 \text{ s}^{-1}$ [28]. In the current work, the rate of ET of the MB-dendrimer films drop even more in the presence of adsorbed rHRP (to $4.1 \pm 0.1 \text{ s}^{-1}$), which may also be connected with the restricted conformational mobility of the dendrimer molecules.

Figure 3. Figure 3. Baseline-corrected cyclic voltammograms recorded in N_2 -saturated 50 mM phosphate buffer solution, pH 7 with **A)** MB/0.2 mM PAMAM/NSTP/Au and **B)** MB/18 mM PAMAM/NSTP/Au modified electrodes (**1**) before and (**2**) after adsorption of rHRP. Potential scan rate was 0.1 V s^{-1}

Adsorption of rHRP on the MB-dendrimer films significantly affected the CV waveform (Figure 3, curve 2): the MB peak potentials shifted in the negative direction, became broader, and showed evident shoulders at the peroxidase heme potentials ($-293 \pm 2 \text{ mV}$, as estimated from de-convoluted CVs in Figure 3). Therefore, the ET reactions of both rHRP and MB appear to be mutually affected [NB: Under these conditions it was not possible to calculate the enzyme's true k_s value, since the de-convoluted data were not credible enough (peak potential values) to be used for analysis of the k_s values].

From the change in the CV peak areas the amount of rHRP deposited on the surface was estimated as $20 \pm 2 \text{ pmol cm}^{-2}$, which is in good agreement with the above obtained values for rHRP adsorbed on dendrimer films. At this point it should be noted that both PAMAM and MB, as well as HRP are overall positively charged at pH 7 (HRP's isoelectric point is 8.9) [34]. However, HRP has some surface amino acid residues bearing carboxylic acid groups ($pK_a \sim 2\text{--}4$, thus negatively charged at pH 7) and residues with a large number of oxygen- and nitrogen-containing functionalities. Therefore, the driving force for the enzyme to interact with the nanostructured dendrimer-modified surface seems to be a complex combination between hydrogen bonding ($N\text{--}H \cdots O$ and $N\text{--}H \cdots N$) and electrostatics, producing nevertheless strong binding between the enzyme and the dendrimer-modified surface. A very stable adsorption of electrochemically active rHRP on the MB/dendrimer-electrodes was observed, with 97% of the initial response kept after three days storage (Figure S3).

The bioelectrocatalytic wave of H_2O_2 reduction by rHRP adsorbed on the MB-dendrimer electrodes started from $\sim 0 \text{ V}$ (Figure 4). The disappearance of the bioelectrocatalytic signals associated with the Compound I/Compound II reduction, at potentials more positive than both HRP's Fe^{3+}/Fe^{2+} and

dendrimer's MB redox couples, could be followed as well. This inherent HRP's reaction (1) was only observed for rHRP adsorbed on unlabeled-dendrimer films, *i.e.* when the enzyme was in direct electronic communication with the electrode (Figure 2). The disappearance of the signals from reaction (1) in the MB-dendrimer system can be thus considered as another evidence of the rHRP bioelectrocatalysis mediated by the MB-dendrimer wires. It should also be noted that the apparent bioelectrocatalytic efficiency of rHRP was not affected by the dendrimer surface concentration (Figure 2 and 4) and, therefore, further studies were done only with diluted layers.

Figure 4. Representative cyclic voltammograms of rHRP electrostatically adsorbed on **A)** MB/0.2 mM PAMAM/NSTP/Au and **B)** MB/18 mM PAMAM/NSTP/Au in the (1) absence and (2) presence of 0.1 mM H₂O₂ in N₂-saturated 50 mM PBS, pH 7, at a scan rate of 5 mVs⁻¹. Insets: CVs recorded for rHRP/MB/PAMAM/NSTP/Au at scan rates of 10, 25, 50, 70, 90, 100, 125, 150, 200 mVs⁻¹ and dependence of the MB CV peak current on the potential scan rate (v). $\Gamma_{\text{rHRP/MB/PAMAM/NSTP/Au}} = 20 \pm 2$ pmolcm⁻² estimated as a difference $\Gamma_{\text{HRP}} = \Gamma_{\text{HRP+MB}} - \Gamma_{\text{MB}}$.

Chronoamperometric detection of H₂O₂ was carried out with the rHRP-MB-dendrimer film at an applied potential of -0.28 V, and the electro-enzymatically catalyzed reduction of H₂O₂ was analyzed in terms of Michaelis-Menten model (Figure S4). The value of the apparent Michaelis-Menten constant (K_m) was determined to be 35 μ M, which is comparable to the K_m determined for other HRP-electrode systems, such as 160 μ M shown for HRP on Nafion-cysteine/Au electrode [52] and 59 μ M obtained on Fe₂O₃ nanoparticles modified pyrolytic graphite [53].

Based on the obtained results, we can conclude that despite the advantageous reagentless approach for bioelectrocatalysis, the electrocatalytic activity of rHRP wired by MB-dendrimers does not offer pronounced advantages over the direct ET approach. It may be connected with a specific balance of different bioelectrocatalytic reaction steps that involve, in the case of MB-labeled dendrimers, an extra step of interactions between the MB pendants fixed in their quite robust positions on the dendrimer periphery and the rHRP molecules. The absence of the sufficient flexibility of the MB redox groups, compared to the freely diffusing mediators, might introduce an additional barrier in ET reaction between the heme – MB – and the electrode surface. It may also be connected, though to a lesser extent, with insufficient electrostatic compatibility between the positively charged HRP and the positively charged MB-dendrimer matrix. In the case of one-cofactor containing heme enzymes such as HRP, whose direct electrochemistry and bioelectrocatalysis could be followed on a variety of electrode materials [8], the bio-mimicking environment such as that provided by dendrimers may not be the main “must-have” condition for their pronounced bioelectrocatalytic function.

3.3. ET reactions of SO_x at PAMAM/NSTP/Au and MB/PAMAM/NSTP/Au electrodes

Complex heme-containing sulfite oxidase (SO_x) is a dimeric enzyme that contains a molybdopterin catalytic site, at which oxidation of sulfite occurs, and a heme *b* cofactor that accepts electrons from the Mo^{IV} complex and passes them further to the external electron acceptor such as cytochrome *c* (Scheme 1). In the enzyme resting state the Mo active center is about 32 Å away from the electron acceptor heme, this distance being far too much for intramolecular electron tunneling [54]. However, SO_x contains a flexible polypeptide loop that allows the enzyme to switch conformation and shorten the ET distance during the catalytic transformation of sulfite. This conformational switching depends very much on the nature of anions present in solution, such as divalent inorganic ones that can inhibit the interdomain ET reaction and thus, the overall bioelectrocatalytic activity of SO_x [55].

Scheme 1. General scheme of the mechanism of SOx biocatalytic oxidation of sulfite [56].

It is important to highlight that SOx needs a bio-mimicking environment and a proper orientation on the modified electrodes, specifically, through the negatively charged heme-containing domain. This heme-containing domain operates as a relay between SOx's catalytic domain and the external electron acceptor (e.g. an electrode modified with positively charged SAMs) [57]. The only possible bioelectrocatalysis pathway is *via* the heme relay, and no external mediation between the molybdopterin domain and the electrode is possible. Thus, the positively charged unlabeled and MB-labeled dendrimer layers were expected to provide the sought electrical and environmental medium for bioelectrocatalysis of SOx entrapped into such layers.

Electrostatic adsorption of SOx on the unlabeled dendrimer monolayer resulted in its pronounced direct electrochemistry, exhibiting a couple of redox peaks centered at -185 ± 6 mV (Figure 5A, inset). This correlates fairly well both with the formal redox potential of SOx's heme of -115 mV, previously determined by spectrophotometric analysis of the redox transformation of the SOx heme domain on aldrithiol SAMs [55], and with the redox potential of Mo(VI/V) of -110 mV at pH 8 (90 mV *vs.* SHE) [58]. Therewith, cathodic and anodic peaks were asymmetrical, with the anodic peak exhibiting a shoulder that may be assigned to different populations of enzyme molecules undergoing ET in different orientations (*i.e. via* the molybdopterin domain and *via* the heme domain), similar to earlier observations by Armstrong and coworkers [58]. Here, we suggest that the dominating couple of peaks may be ascribed to the heme signal.

Figure 5. Bioelectrocatalytic CVs of SOx electrostatically adsorbed on **A)** unlabeled and **B)** MB redox labeled 0.2 mM PAMAM/NSTP/Au recorded at a scan rate of 2 mVs⁻¹ in N₂-saturated 0.1 M Tris-HCl, pH 7.4, in the presence of 0, 16, 33, 66, and 132 μM Na₂SO₃. Insets show the baseline-corrected CVs (2) before and (1) after SOx immobilization, CVs recorded at 1 Vs⁻¹.

From the peak widths at mid-height the number of electrons involved in the ET reaction was determined to be 1.1 ± 0.2 (Table 2). The surface concentration of electroactive SOx was about 0.25 ± 0.02 pmol cm⁻², comparable to the 0.45 ± 0.5 pmol cm⁻² previously observed for SOx adsorbed on cysteamine-modified gold surface and entrapped under a membrane [57]. The k_s was 14.5 ± 0.4 s⁻¹ calculated from the peak-to-peak separation according to the Laviron theory; this k_s falls in the range of values reported for SOx on other positively charged surfaces, such as 1-mercapto-1-undecanol and 11-mercapto-1-undecanamine mixed alkanethiol layer (15.2 ± 0.3 s⁻¹) or cysteamine (13.8 ± 1.5 s⁻¹) [57]. Overall we can conclude that the positively charged PAMAM-modified electrodes provide a compatible microenvironment for SOx immobilization that also favors the ET reaction between the heme domain and the electrode.

Table 2. The formal potential of heme (E^0), the amount of enzyme on the electrode surface (Γ_{enzyme}), the apparent heterogeneous electron transfer rate constant (k_s), and the number of electrons transferred during the ET (n).

	0.2 mM PAMAM/NSTP/Au				MB/0.2 mM PAMAM/NSTP/Au		
	E^0 , mV ^(a)	Γ_{enzyme} , pmol cm ⁻² (b)	k_s , s ⁻¹	n	E^0 , mV ^(a)	Γ_{enzyme} , pmol cm ⁻² (b)§	k_s , s ⁻¹

rHRP	- 341±7	17±2	2.8±0.2 ^(d)	0.78±0.03	-293±2	20±2	—
SOx	-185±6	0.25±0.02	14.5±0.4 ^(c)	1.1±0.2	—	0.35±0.05	—
^(a) E^0 vs Ag/AgCl (3 M KCl) at pH 7. ^(b) $\Gamma_{enzyme} = \frac{Q}{nFA_{electroactive}}$ ^(c) $\Gamma_{enzyme} = \Gamma_{enzyme+MB} - \Gamma_{MB}$. k_s values were calculated at ^(c) 1 V s ⁻¹ and ^(d) 0.2 mV s ⁻¹ .							

Bioelectrocatalysis of sulfite oxidation by SOx was probed in 0.1 mM Tris-HCl, pH 7.4 (known not to inhibit the internal ET reaction of SOx [55]) and low scan rates, giving enough time for the catalytic cycle to be accomplished (2 mV s⁻¹). Our experiments in PBS, as expected, did not show any bioelectrocatalytic activity of SOx. In the absence of a mediator, the catalytic currents are observed upon addition of sulfite starting from the potentials of the redox center of the enzyme (Figure 5A, region II). Although the nonspecific oxidation of sulfite at the electrode could also be followed (Figure S6 and Figure 5A, region III) it starts at much more positive potentials and therefore can be easily distinguished from bioelectrocatalysis itself. The third redox process that can be followed at more negative potentials is the cathodic reduction of oxygen catalyzed by SOx's heme (Figure 5A, region I), typical of electrode-wired heme enzymes when oxygen (even trace amounts as in our case) is present in solution [6]. That somehow supports our assumption on the dominating contribution from the heme to the detected non-catalytic CV signals in Figure 5A, inset. The O₂ reduction reaction catalyzed by SOx's heme was competitively inhibited in the presence of the oxidized substrate, sulfite. The apparent catalytic efficiency of sulfite oxidation by SOx, estimated from the ratio between the catalytic and non-catalytic peak currents, was 2.2.

When SOx was immobilized on MB-labeled PAMAM layers, the height of the voltammetric peaks correlating with both the SOx and MB responses increased, and shoulders appeared at potentials less negative than the MB peaks alone (Figure 5B, inset). Interestingly, neither MB nor SOx peak potentials significantly change, which might be correlated with quite similar potentials of SOx's heme (-185 mV) and MB covalently attached to dendrimers (-189 mV) – and a very small fraction of SOx wired (in contrast, to rHRP, which surface coverage was only twice lower than the monolayer). Therewith, the shoulders in CV better fit the potentials of the molybdopterin center, though, considering the complexity of the system, it is difficult to unambiguously judge on that.

In the presence of sulfite, its catalytic oxidation by MB-wired SOx could be followed from the potentials of MB redox transformation (*i.e.* from ca. -200 mV) and very quickly reached saturation, with limiting oxidation currents of 0.4 $\mu\text{A cm}^{-2}$ (Figure 5B). If analyzed in terms of relationship

between the non-catalytic and catalytic limiting currents, the apparent catalytic efficiency of sulfite oxidation by MB-wired SOx was 11, which was essentially higher than the bioelectrocatalytic performance of SOx on unlabeled PAMAM layers. It is worth to note, that for SOx wired by MB-dendrimers, no electrocatalytic reduction of oxygen could be followed at potentials more negative than SOx's heme (Figure 5B), in contrast to the directly wired SOx (Figure 5A). Since MB itself does not catalyze O₂ reduction, we may conclude that no direct ET contact between the heme and the electrode occurred for the MB-wired SOx, and when there is no direct contact – no O₂ catalytic activity is displayed by the heme. Those results on bioelectrocatalysis of SOx mediated by MB-labeled dendrimers appeared to be much promising compared to rHRP-dendrimer systems, not the least due to the absolute necessity of electrostatic compatibility between the wire and the negatively charged heme domain of SOx and bio-mimicking environment necessary for the electrode function of this complex, intermembrane enzyme.

4. Conclusions

To conclude, two heme-containing enzymes, one-cofactor heme-containing HRP and heme- and molibdopterin-containing SOx, were electrically wired to electrodes by MB-labeled PAMAM dendrimers. The efficiency of wiring, in terms of the amount of enzyme wired and the efficiency of bioelectrocatalysis, depended essentially on the nature of the enzyme studied. In the case of native glycosylated HRP, no wiring was observed, while with de-glycosylated recombinant rHRP both pronounced direct ET-based and MB-wired electrochemistry of rHRP's heme can be followed, not the least, due to the presence of multiple ET pathways within the peroxidase molecular structure [11]. Therewith, the bioelectrocatalytic performance of those two systems was comparable, with some advantages demonstrated by the direct ET-based bioelectrocatalysis of rHRP entrapped in the PAMAM dendrimer monolayer on gold.

In the case of complex cofactor SOx, in which its heme just relays the electron flow between the electrode and the catalytic domain of SOx, the MB/dendrimer wiring of SOx (and bioelectrocatalysis) was much more efficient compared to direct electrochemistry of SOx on the PAMAM-modified electrodes. It is plausible that the presence of the multiple, positively charged MB redox groups attached to the periphery of the dendrimer and relaying the electron transfer over the dendrimer surface, provide statistically larger number of the electrostatically compatible sites for the electrical contact with the negatively-charged heme domain of SOx (compared to the restricted number of orientations of SOx on the electrode surface *via* the heme domain that favor direct ET). The overall

PAMAM nanostructures were shown to additionally stabilize the enzyme integrity on the electrodes. In this context, redox-labeled dendrimers can be considered as an advanced tool for studies of ET properties and achievement of bioelectrocatalysis of complex intermembrane and membrane enzymes, whose direct electrochemistry and bioelectrocatalysis can be impeded by their either improper orientation or insufficient stability on electrodes, in the absence of a biomimicking medium required for their electrocatalytic function.

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