Kinetic analysis of horseradish peroxidase “wiring” in redox polyelectrolyte–peroxidase multilayer assemblies

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Abstract

A hydrogen peroxide sensitive interface based on a self-deposited redox polyelectrolyte–peroxidase assembly was used as model system for investigation of the electron transfer between chemically modified horseradish peroxidase and the redox active centers of poly[(vinylpyridine)Os(bpy)2Cl] redox polymer. The assembly was built up by sequential adsorption of the redox polymer and the peroxidase on gold electrodes modified with a self-assembled monolayer of 3-mercapto-1-propane sulfonic acid. Making use of a kinetic model, formulated for steady-state conditions, and of the rotating disc electrode technique it was possible to estimate: (i) the second order rate constant of reaction between the modified peroxidase and hydrogen peroxide ($2.74 \times 10^5$ M$^{-1}$ s$^{-1}$) and (ii) the apparent turnover number of the oxidized enzyme regeneration by the osmium redox center (2.14 s$^{-1}$), i.e., two parameters that reflect the efficiency of the electrical communication within the investigated interface.

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1. Introduction

Amperometric biosensors based on peroxidase-modified electrodes have been developed for detection of hydrogen peroxide and organic hydroperoxides, as well as phenols, aromatic amines, potassium cyanide, etc., i.e., of molecules that are substrates, activators, or inhibitors of the enzyme catalyzed reaction [1]. On the other hand, peroxidase-modified electrodes can be used for the immuno-detection of analytes [2–4] or can be coupled with different hydrogen peroxide producing oxidases resulting in sensors for many other analytes of interest [5–8]. Horseradish peroxidase (HRP, EC 1.11.1.7.) – the model enzyme for whole class – is the most commonly used peroxidase for bioelectrode construction.

Electrodes modified by coverage with redox or electron conducting polymers, incorporating physically or chemically attached enzyme molecules, represent an important and extensively studied group of peroxidase-based electrodes [1]. Thus, electrodes based on HRP, bound to [OsIII(bpy)2Cl]-containing polyvinyl pyridine or polyvinyl imidazole [9–11], are among the most successful peroxidase-based modified electrodes, if such criteria as sensitivity, lower detection limit and stability are considered (for comparison see Table 2 in reference [1]).

The above-mentioned polymer materials are commonly referred to as redox “wire” or redox hydrogels. Redox “wire” is a redox macromolecule designed to: (i) physically attach the enzyme to the electrode; (ii) strongly complex the enzyme protein; (iii) electrically connect the enzyme’s redox center to the electrode, all these without serious deactivation of the enzyme [12]. When an oxidoreductase is integrated in such a three-dimensional polymer matrix, electrons are transported via the redox polymer network between the enzyme and redox polymers is now available [9,12–15], though
the subject is far to be exhausted. Thus, of particular interest are kinetic aspects of the enzymes “wiring” by redox polymers [12].

Recently it was demonstrated that uniform thin polymeric films can be assembled on a variety of substrates by alternating electrostatically controlled adsorption of polycations and polyanions on charged surfaces [16]. In a short time, layer-by-layer assembly technique proved to be an efficient strategy for the fabrication of multicomponent nanostructured films on various solid supports [17].

Much of the recent work on these polyelectrolyte multilayers has focused on generalizing and expanding the technique to various combinations of charged components, including various synthetic polyelectrolytes [17], biopolymers, such as proteins [18,19] and enzymes [20–29]. Most importantly, building-up of multilayer redox polyelectrolyte-oxidoreductase architectures, based on electrostatic interactions, allows fine control of the film thickness and the amount of the biomolecule at the electrode surface and, from these considerations, the technique could present a promising approach to electrode coating with thick redox hydrogel films loaded with enzyme molecules. Nevertheless, only few papers [27,28] dealt with a kinetic analysis of these sensing structures, in order to estimate quantitatively the efficiency of the enzyme electrical “wiring” as well as the main kinetic parameters involved in electrical signal generation. The approach is essentially based on the expression describing the dependence of the catalytic current on the substrate concentration, when the substrate diffusion is neglected [30,31]. Combining the kinetic parameters for the soluble enzyme (the Michaelis–Menten constant and the turnover number) with those furnished by the non-linear fit of the above-mentioned equation to the experimental data, an estimation of the enzyme surface concentration and the pseudo-first-order rate constant corresponding to the enzyme/redox polymer reaction in the investigated architecture was possible. However, it is worthy to mention that this approach disregards the inherent variation of the enzyme kinetic parameters due to its immobilization on the electrode surface.

In a previous paper [8], the potentiality of multilayer self-deposition technique based on electrostatic interactions for the enzyme bioelectrodes design was analyzed. Thus, it was described the use of layer-by-layer self-deposition of a positively charged poly[(vinylpyridine)OsIII/II(bpy)2Cl] redox polymer (RP) partially quaternised with bromoethylamine was synthesized as described elsewhere [32]. Horseradish peroxidase (HRP, EC 1.11.1.7, 290 U mg\(^{-1}\) (solid); type VI) was supplied by Sigma. The enzyme was chemically modified according to procedure described elsewhere [8]. The purpose of the modification was manipulation of the enzyme charge by introducing sulfonate groups covalently bound to the enzyme [8]. The activity of the modified enzyme was nearly the same as that measured before the modification, when calculated per protein content (U mg\(^{-1}\)). The H\(_2\)O\(_2\) solutions were prepared daily by dissolving 30% (w/w) H\(_2\)O\(_2\) (Merck, Darmstadt, Germany) in a phosphate buffer solution. All other reagents were of analytical grade. Aqueous solutions were prepared with water purified with a Milli-Q system (Millipore, Milford, USA).

Electrochemical measurements were performed in a conventional single-compartment three-electrode cell using a computer-controlled voltammetric analyzer AutoLab-PGSTAT10 (Eco Chemie, Utrecht, The Netherlands). The reference electrode was a potassium chloride saturated silver/silver chloride electrode (Ag/AgCl/KCl\(_{sat}\)), to which all the electrode potentials are referred. A coiled platinum wire served as auxiliary electrode. Steady-state amperometric measurements at different rotation speeds of the working electrode were performed using a modulated speed rotator (model AMS-FRX, Pine, Grove City, PA, USA).

Gold disc electrode (Ø 5 mm) was used as working electrode throughout the experiments. The electrode was polished with 0.3 μm α-alumina and 0.05 μm γ-alumina.

The sensitivity of a peroxidase electrode [1] and actually the highest sensitivity ever reported for hydrogen peroxide detection at HRP-based enzyme bioelectrodes [9]. A straightforward way to increase the sensitivity is further alternate deposition of new biocatalytic and transducing layers, resulted in a multilayer assembly. This could bring about an increase of the importance of both electron “diffusion” and the substrate diffusion limitations. Consequently, only a thorough kinetic analysis of the resulted multilayer RP-HRP assembly, as well as its structural elements, could provide further insight into the interface and could be of help in identification of certain criteria for optimization of the bioelectrode response. In this sense, a simple approach for quantitative estimation of the rate of electron transfer between the enzyme active center and the transducing macromolecule, as well as of the immobilized enzyme catalytic activity, is proposed and discussed.
(Buehler, IL, USA) to a mirror-like finish, followed by cleaning for 5 min with ethanol and water in ultrasonic bath. Subsequently, the electrode was etched electrochemically by potentiodynamic cycling (20 mV s\(^{-1}\) scan rate) between +0.44 and +1.5 V for 30 min in 0.1 M sulfuric acid containing 10 mM potassium chloride. During this time, an up to 100 nm thick layer of gold is dissolved by chloroaurate complex formation [33]. The real surface area was estimated from oxygen adsorption measurements, according to the method proposed by Rand and Woods [34].

The negatively charged gold surface (Au/MPS) was prepared by immersing the electrode into 1 mM MPS solution in ethanol for about 12 h, followed by thorough rinse with pure ethanol and re-immersing into the deposition solution for 1 h more. The growth of the Au/MPS/RP/HRP\textsubscript{mod}/RP multilayer assembly was achieved by sequential deposition of two RP layers and one intercalating HRP\textsubscript{mod} monolayer by alternative immersion of the MPS monolayer covered Au electrode into corresponding aqueous solutions. RP was deposited for 2 h from its 100 \(\mu\)g ml\(^{-1}\) aqueous solution at room temperature while stirring vigorously. Biocatalytic layer was obtained by self-deposition of the modified peroxidase (HRP\textsubscript{mod}) for 2 h at 4\(^\circ\)C from 100 \(\mu\)g ml\(^{-1}\) enzyme aqueous solution, prepared in 0.1 M phosphate buffer solution (pH 6.0). Between deposition steps the electrode was rinsed with pure water and then was allowed to dry at room temperature for 30 min. Before deposition of the biocatalytic layer no significant amperometric response at the working potential (0.1 V) was observed upon addition of hydrogen peroxide.

### 3. Kinetic model

In Fig. 1 is depicted the idealized picture of the interface in study. It is commonly accepted that in multilayer assemblies based on electrostatic interactions, regardless of the components’ nature (polyelectrolyte, charged colloidal particles, large organic or inorganic molecules), the deposition is restricted to formation of a nearly single molecular layer [17]. As electrostatic forces are perceived as the forces that control the assembly growth [8], HRP\textsubscript{mod} adsorption ought to be limited to a nearly single molecular layer. In order to place the enzyme molecule into the same environment as that in (RP/HRP\textsubscript{mod}/RP)\textsubscript{n} multilayer film and to eliminate or diminish the enzyme orientation effect on the signal generation, the RP/HRP\textsubscript{mod}/RP assembly was investigated.

The following reaction sequence can be proposed for the case of mediated bioelectrocatalytic reduction of \(\text{H}_2\text{O}_2\) at Au/MPS/RP/HRP\textsubscript{mod}/RP electrode:

\[
\text{HRP}(\text{Fe}^{3+}) + \text{H}_2\text{O}_2 \xrightarrow{k_1} \text{Comp. I} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{Comp. I} + \text{Os}^{\text{II}} (+\text{H}^+) \xrightarrow{k_2} \text{Comp. II} + \text{Os}^{\text{III}} \quad (2)
\]

\[
\text{Comp. II} + \text{Os}^{\text{II}} (+\text{H}^+) \xrightarrow{k_3} \text{HRP}(\text{Fe}^{3+}) + \text{Os}^{\text{III}} + \text{H}_2\text{O} \quad (3)
\]

\[
(\text{Os}^{\text{II}})_1 + (\text{Os}^{\text{III}})_2 \xrightarrow{k_e} (\text{Os}^{\text{III}})_1 + (\text{Os}^{\text{II}})_2 \quad (4)
\]

\[
\text{Os}^{\text{III}} + \text{e}^{-} \xrightarrow{k_{5\text{H}}} \text{Os}^{\text{II}} \quad (5)
\]

![Fig. 1. Schematic representation of the investigated interface.](image-url)
where HRP(Fe³⁺) is the ferrie enzyme; Comp. I and Comp. II are oxidized intermediates (the so-called compounds I and II [35]); [Os] and [OsIII] designate the reduced and, respectively, oxidized forms of [OsIII(H(bpy),Cl] complex attached to the polymer matrix. Reaction (1) involves a two-electron oxidation of the ferrihaem prosthetic group of the enzyme by H₂O₂. The resulted intermediate consists of oxyferryl iron (Fe⁴⁺=O) and a porphyrin π cation radical [35]. As a result of two consecutive one-electron transfers from the electron donor substrate (OsIII), the enzyme, after passing through an intermediate species (Comp. II), is returned to its resting state. Reaction (4) describes transport of the oxidizing equivalents to the electrode surface by the self-exchange reaction (electron hopping). Rapid electron transfer from the electrode surface to the oxidized mediator closes the electrocatalytic cycle.

In case of rotating disc electrode (RDE) technique, H₂O₂ reduction current measured at the Au/MPS/RP/HRPmod/RP electrode is a combination of the mass-transport-limited current Iₖ and the reaction-rate-limited current Iₖ [36]:

\[
\frac{1}{I} = \frac{1}{I_k} + \frac{1}{I_e}.
\]  

(6)

Theoretically, the mass-transport-limited current could consist of two components: one characterizing the substrate diffusion in solution (through the diffusion layer) and the other one – through the external RP layer. Though, as it will be shown in Section 4 the term describing the last component can be disregarded.

In accordance with the Levich equation [36], the mass-transport-limited current is a function of the hydrogen peroxide bulk concentration [H₂O₂] and the angular rotation speed ω of the electrode:

\[
I_k = 0.62nFAD^{2/3}ω^{1/2}v^{-1/6}[H₂O₂],
\]  

(7)

where n is the number of electrons transferred to the enzyme in one catalytic cycle (theoretically n = 2); F is the Faraday constant; A (cm²) is the geometric area of the electrode; D (cm² s⁻¹) is the hydrogen peroxide diffusion coefficient; v (cm² s⁻¹) is the kinematic viscosity of water; [H₂O₂] (mol l⁻¹) is the hydrogen peroxide concentration.

For the interface in study the kinetic limitations are not obvious, but, based on comparative analysis and experimental tests, reactions (1)–(3) were identified as the most probable rate-limiting steps. Firstly, the rate of reaction between native HRP and H₂O₂ is known to be high (15 × 10⁶ M⁻¹ s⁻¹ [37]). Though effective in the enzyme immobilization matrix [38], as well as possible effect of the enzyme modification, could bring about a significant decrease of the enzyme activity. Secondly, [OsIII(bpy),Cl] complex reacts slowly with oxidized native HRP in solution (7.8 × 10² M⁻¹ s⁻¹ [10]), being a quite sluggish reductive substrate for HRP. As it will be shown in Section 4 the rate of heterogeneous electron transfer between the polymer’s redox centers and electrode surface as well as electron “diffusion” through the transducing polymer can be disregarded as rate limiting steps. Based on these considerations, it was assumed that kinetic limitation comes from reactions (1)–(3) only.

Formally, in case of the investigated system the reaction-rate-limited current can be expressed analogously to that for mediated bioelectrocatalytic reduction of H₂O₂ at (sub)monolayer HRP-modified electrode in presence of a freely diffusing electron donor substrate [39]:

\[
\frac{1}{I_k} = \frac{1}{2n_FAD^{2/3}ω^{1/2}v^{-1/6}[H₂O₂]} + \frac{1}{k_{SET}}.
\]  

(8)

where \(I_k\) (mol cm⁻²) is the surface concentration of the modified enzyme; [Os] (mol l⁻¹) is concentration of the Os redox centers attached to the polymer matrix formed by the two RP transducing layers; n₁ is the number of electrons transferred per each electron donor species (n₁ = 1). This approach is quite simplistic as it is unlikely the Os redox centers within the RP layers adopt a uniform three-dimensional distribution.

In spite of those, the model allows estimation of the turnover number of the oxidized enzyme reduction by [OsIII(bpy),Cl] redox centers. Thus, Eq. (8) can be rewritten as follows:

\[
\frac{1}{I_k} = \frac{1}{2n_FAD^{2/3}ω^{1/2}v^{-1/6}[H₂O₂]} + \frac{1}{k_{SET}}.
\]  

(9)

The (apparent) turnover number \(k_{SET}^{-1}\) could be informative from at least two reasons: (i) it presents a parameter characterizing the efficiency of the electrical communication between enzyme and redox polymer, and, consequently, could be used as a criterion for optimization of the bioelectrode response; (ii) it allows direct comparison with other peroxidase-based bioelectrodes, particularly those based on direct electron transfer (ET) between the enzyme active center and the electrode surface.

By combining Eqs. (6) and (7) the Koutecky–Levich equation for the observed bioelectrocatalytic H₂O₂ reduction current can be derived:

\[
\frac{1}{I} = \frac{1}{I_k} + \frac{1}{0.62nFAD^{2/3}ω^{1/2}v^{-1/6}[H₂O₂]}.
\]  

(10)

By plotting \(I^{-1}\) versus \(ω^{-1/2}\) the reaction-rate-limited current can be separated from the mass-transport-limited one. From the slope and intercept of the dependence of \(I_k\) on H₂O₂ concentration in doubly reciprocal plot, \(k_{SET}\), respectively, can be calculated.

It is worth to mention that the Koutecky–Levich formalism requires both diffusional and kinetic processes to be of the first order. This is not the case of the mechanism of H₂O₂ reduction at the interface in study: Eq. (9) is non-linear in hydrogen peroxide concentration.
and, consequently, Eq. (10) is not strictly correct. Though, in conditions of the enzyme under-saturation the kinetic process is of the first order in hydrogen peroxide concentration (see also Section 4). Consequently, the application of the Koutecky–Levich treatment is justified.

### 4. Results and discussions

As it can be seen from Fig. 2 the second RP layer is electrically connected to the electrode surface, thus forming with the first RP layer a more or less continuous redox network. In accordance with the data presented in Table 1, the electrochemical behavior of the two RP layers as a whole presents somehow unexpected characteristics when compared to that of the first RP layer. Thus, the binding of the second layer of redox polymer does not double the electrode coverage by redox active centers. It results that the amount of redox polymer in the outer layer is not enough to assure a full sandwiching of the HRP molecules between two redox polymer layers. Consequently, the transduction efficiency of the investigated architecture will be diminished. In the same time, the presence of the second redox polymer layer slightly increases the peak separation ($\Delta E_p$) as well as the peak width at half maximum ($E_{\text{FWHM}}$). This behavior could be assigned partially to the manifestation of electron hopping mechanism of the charge transport through the polymer network formed by the two RP layers. However, the electron diffusion pathways are too short to make an important difference to the surface-confined redox couple behavior. On the other hand, the interposed enzyme layer could also exert an effect on the RP electrochemical response.

On the other hand, the electrochemical parameters values ($\Delta E_p$ and $E_{\text{FWHM}}$) of both voltammograms present relatively small discrepancies in comparison with their theoretical values. These differences should reflect the interactions (attractive for the anodic wave, and repulsive for the cathodic one) expected to exist between the redox active centers and their microenvironment.

As it was stated above, heterogeneous electron transfer was disregarded as a possible rate-limiting step. This assumption is supported by the fact that observed electrocatalytic current (in conditions of high rotation speed and at saturating hydrogen peroxide concentrations) does not depend on the applied potential in the range between 0 and 300 mV (results not shown). This is not surprising at all as the redox couple is known to be fast.

$\text{H}_2\text{O}_2$ reduction currents measured at the investigated bioelectrode at different $\text{H}_2\text{O}_2$ concentrations and rotation speeds were plotted in Levich coordinates (Fig. 3). It was concluded that the system in study shows a well-behaved mixed control (diffusional-kinetic) current response. From Fig. 4 it can be seen that increase of the $\text{H}_2\text{O}_2$ concentration brings about decrease of the slope of the Koutecky–Levich plot and, consequently, decrease of the mass-transport-limited current contribution to the observed current up to negligibly small values.

The slope of the Koutecky–Levich plot depends on the bulk concentration of $\text{H}_2\text{O}_2$ and is proportional to the number of electrons transferred per $\text{H}_2\text{O}_2$ molecule (see Eq. (7)). The experimental number of electrons (1.93), calculated from the slope of the Koutecky–Levich plot versus $[\text{H}_2\text{O}_2]$ (linear least-square regression analysis, Fig. 5(A)), is close to the theoretical value of 2. Hence an important criterion of the Koutecky–Levich treatment applicability to the investigated system is

<table>
<thead>
<tr>
<th>Interface</th>
<th>$E^0$ (mV)</th>
<th>$\Delta E_p$ (mV)</th>
<th>$E_{\text{FWHM}}$ (mV)</th>
<th>$I_{\text{pa}}/I_{\text{pc}}$</th>
<th>$\Gamma \times 10^{10}$ (mol cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au/MPS/RP/</td>
<td>324</td>
<td>14</td>
<td>76</td>
<td>108</td>
<td>1.41</td>
</tr>
<tr>
<td>Au/MPS/RP/HRP_mod/RP</td>
<td>333</td>
<td>18</td>
<td>88</td>
<td>119</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*See Fig. 2 for experimental conditions.
fulfilled. It is worth mentioning that results of the Student’s test ($t_{tab} = 3.338, t_{calc} = 3.499$, significance level 0.01, $k = 7$) indicate that the intercept of the regression line is not significantly different from zero. Consequently, the current characterizing the hydrogen peroxide diffusion through the outer RP layer does not have any important contribution to the mass-transport-limited current [40].

In Fig. 5(B) the intercepts of the Koutecky–Levich plot ($I_K^{1/2}$) are plotted against $[\text{H}_2\text{O}_2]^{-1}$. In accordance with Eq. (9), estimation of both $k_1$ and $k_{S,ET}$ is possible provided that the surface concentration of HRP$_{mod}$ is known. In this paper the enzyme surface concentration $I_{HRP}$ was approximated to $8\ \text{pmol cm}^{-2}$, corresponding to the closely packed HRP monolayer (HRP is a globular protein of about 50 A diameter [41]). We expect this value to be an overestimation of the enzyme surface concentration. This approximation induces an important uncertainty in estimation of both rate constants. However, this drawback could be overcome by experimental estimation of the peroxidase surface concentration, making use of such a sensitive gravimetric technique as quartz crystal microbalance (14 pmol cm$^{-2}$, on a graphite coating [42]) or from UV-vis spectra of the fluorescein-labeled HRP (1.2 pmol cm$^{-2}$, in a multilayer assembly formed by layer-by-layer deposition of concanavalin A and HRP [43]). Another related problem, of fundamental importance, is the enzyme layer structure (monolayer vs. multilayer adsorption) and the fraction of the catalytically active enzyme effectively connected to the electrode. In Section 3 we argue for a monolayer deposition of the enzyme, but that is the only information that we can provide on the enzyme layer structure and composition for the moment (see Fig. 1).

Using the above-mentioned approximate value for $I_{HRP}$, the rate constant $k_1$ of the reaction between $\text{H}_2\text{O}_2$ and HRP$_{mod}$ was found to be $2.74 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and the apparent turnover number $k_{S,ET}$ was equal to $2.14 \text{ s}^{-1}$. As it was mentioned above, the non-linearity of Eq. (9) in hydrogen peroxide concentration could be a source of the errors in estimation of the rate constants. The errors induced should probably be small, as the experimental points at lowest, i.e., the enzyme under-saturating, concentrations are dominating ones in the linear regression analysis.

The rate constant $k_1$ for HRP$_{mod}$ in RP/HRP$_{mod}$/RP architecture is about 54 times lower than that for the wild type HRP in solution ($15 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [37]). This considerable difference could be assigned to the enzyme immobilization effect and probably to the enzyme partial deactivation upon its chemical modification.

It is worthy noting that, $k_{S,ET}$ allows a direct comparison between the investigated transducing scheme and that based on direct ET between the enzyme active center and electrode. Thus, the value calculated for HRP$_{mod}$ (2.14 s$^{-1}$) is close, but somehow higher than, to the turnover number of the heterogeneous electron transfer for wild type HRP adsorbed on spectroscopic graphite (0.66 s$^{-1}$ [44], pH 7) and is considerably higher than that at a graphite coating (2.0 $\times$ 10$^{-4}$ s$^{-1}$ [34], pH 6.4). In this sense to be also compared the sensitivity of Au/MPS/HRP$_{mod}$/interface (58.1 mA M$^{-1}$ cm$^{-2}$ [8]) and that of pyrolytic graphite electrodes modified with physically adsorbed native HRP (60 mA M$^{-1}$ cm$^{-2}$ [45]), in both cases the enzyme being most probably presented in a nearly single molecular layer. These facts suggest that the
efficiency of the peroxidase regeneration by the transducing redox polymer is most probably superior but still comparable to that provided by direct electron transfer.

At the same time, to be recalled and emphasized the fundamental advantage of using redox polymer networks. These conducting immobilization matrices provide electrical communication for enzyme molecules situated far from the surface, effectively increasing the “virtual” surface of the electrode [46].

5. Conclusions

New results on the electrical communication in redox polyelectrolyte–peroxidase assemblies, built up by electrostatically controlled self-deposition technique were presented and discussed.

It was shown that, using a simple kinetic model, formulated for steady-state conditions, it was possible to estimate the immobilized horseradish peroxidase activity as well as the rate of the oxidized peroxidase regeneration by the osmium redox polymer, expressed by the apparent turnover number of the reaction. The last constant could be useful for investigation of the factors controlling the electrical communication between the enzyme and the transducing macromolecule redox centers, eventually serving as a criterion for optimization of the bioelecrode response.

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