

Laccase Spontaneous Adsorption Immobilization: Experimental Studies and Mathematical Modeling at Enzymatic Fuel Cell Cathode Construction

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Abstract. The activity of a bioelectrode is largely determined by the amount of enzyme adsorbed on its active layer, including the distribution of enzyme along thickness in the carrier layer. The distribution of enzyme is also required for calculations of the characteristics of bioelectrocatalysis process using a mathematical model. In the present article, on the basis of conducted experimental research a mathematical model of laccase immobilization by spontaneous adsorption on carbon-based sorbents of different nature was developed. The model can be used to predict adsorption value and enzyme distribution in the layer of an adsorbent. The model includes the equations of the enzyme concentration changing due to its adsorption on the surface of the carbon material (CM) and the enzyme diffusion over the thickness of CM. The diffusion equation is based on the second Fick's law and contains fractional derivatives instead of the first order derivative.

1. Introduction

More recently, enzymatic electrodes have increasingly been considered by researchers as a possible alternative to electrodes based on noble metals. Enzyme-based systems operate under mild conditions, such as ambient temperature and neutral pH, and with renewable biocatalysts and wide variety of fuels. But, on the other hand, electrochemical characteristics of such systems are not high enough and due to this fact the question of development of highly active biocatalyst systems still remains of current interest. The capability of predicting bioelectrocatalysis characteristics and output for the devices based on bioelectrocatalysis as well as reducing the number of laboratory studies drives research interest to a mathematical modelling of such systems.

By analyzing the researches available at the moment it can be noticed that the activity of immobilized enzymes largely depends on the method of immobilization and on the nature of the adsorbent. While constructing a biofuel cell electrode highly disperse carbon based materials (CM) as carriers [1]-[3] and physical adsorption, covalent binding and entrapment as immobilization methods [4]-[11] are widely used.

Larger, comparing to nanoparticles of metals, molecules of enzymes provide lower values of current density, thus making it necessary to find a method which would increase the degree of filling of



carrier's surface by an enzyme. In addition to that, the electrocatalytic activity of enzymes largely depends on its orientation, so studying possible factors that would increase a percentage of active enzymes on a carrier is important [12]. Immobilization by spontaneous adsorption is of particular interest, since it is assumed that in this case immobilized enzyme is being attached to the electrode in orientation which is favourable for the direct electron transfer from the electrode to the active centers (i.e. enzyme is being attached in active state).

Basic blocks which are used in extended mathematical models of biofuel cells [13]-[21] can be subdivided into the following parts: describing kinetics of the reactions that are taking place in the system, mass and charge balances, initial and boundary conditions. Most of mathematical models available in relevant literature operate with the value of surface concentration of enzyme, but the value is assumed to be equal to the total amount of enzyme deposited on the electrode. With this assumption, it is impossible to define the factors that affect final characteristics of enzymatic electrode.

Considering everything stated above, the main task of this study was to develop a mathematical model which describes the process of spontaneous adsorption of immobilized enzyme on carbon-based materials and to define the factors which affect adsorption process.

2. Materials and methods

This work is a continuation of earlier studies on the influence of the nature of CM on the laccase activity in the reaction of electroreduction of oxygen to water [12].

In this study the *Trametes versicolor* laccase obtained according to the procedure of Shteinberg et al [22] was used. The initial concentration of enzyme was 19 mg/ml with activity $0.189 \times 10^{-3} \text{ mmol ABTS} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. 0.2 M phosphate-acetate buffer solution (pH 4.0-4.5) prepared from $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, acetic acid (CP type) and deionized water were used.



Figure 1. Working electrode with CM deposited on its surface.

The study was carried out on a floating electrode (figure 1) in the form of a tablet made from hydrophobized carbon black, which contained 35% mas. fluoroplastic and was obtained by heat treatment (300 °C) of mixture of acetylene black and fluoroplastic emulsion (containing surfactant), which was placed in 1 cm diameter mold and pressed at a pressure of 5 MPa/cm². At the same time, platinum mesh was pressed into the tablet to be used as current collector. Electrode thickness was equal to 2 mm, the surface area of contact with electrolyte was equal to 1 cm². Electrode of this design by simulating gas diffusion electrode provides efficient supply of oxygen when studying highly dispersed catalytic materials [22] and allows to eliminate the use of binders in electrode-catalyst system which are required in case rotating disc electrode is used.

Commercial carbon black (XC-72) and carbon nanotubes (CNT) provided by D.I. Mendeleyev University of Chemical Technology of Russia were used as CMs. CM was deposited on the surface of the floating electrode, the electrode then was pressed at 5 MPa for 30 s. After that, for the immobilization by spontaneous adsorption the prepared electrode was held for 2 hours on the surface of electrolyte containing laccase in concentration of 0.076 mg/ml. The measurements were conducted after rinsing the electrode in the buffer solution in order to remove the enzyme molecules which are not attached to the electrode surface. Polarization curves were obtained in oxygen atmosphere.

Electrochemical measurements were conducted in a three-electrode cell with separated electrode spaces. A saturated Ag/AgCl electrode was used as a reference electrode and platinum wire was used as a counter electrode. Polarization curves were registered by potentiostat IPC-Pro 3A.

The amount of laccase in solution was determined spectrophotometrically by Specord M40 spectrophotometer with the help of calibration plot of the dependence of the optical density of the ABTS solution on the concentration of laccase solution added. To create a calibration line, 500 μl of ABTS solution was placed into 3mm cuvette, 1 μl of enzyme solution of known concentration was added and the change in optical density was registered at the wavelength of $\lambda = 420 \text{ nm}$ ($\varepsilon_{\text{ABTS}} = 36 \text{ mM}^{-1}\text{cm}^{-1}$) for 6 minutes (time required to reach maximum optical density). The amount of laccase adsorbed on the electrode was calculated as the difference between the initial amount of laccase in the solution and in the solution after enzyme adsorption on CM.

3. Mathematical simulation

3.1. Model of laccase adsorption from solution

The developed mathematical model assumes two modeling domains. The first modeling domain is the volume of the laccase solution and the second one is the active layer of the carbon material (figure 2).

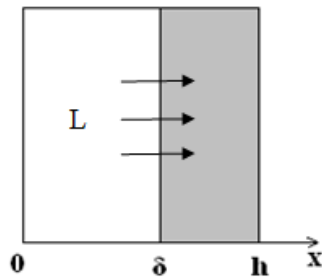


Figure 2. Schematic representation of modeling domains: $0 < x < \delta$ – volume of the laccase solution; $\delta < x < h$ – active layer of CM.

The area of the active layer is CM, which, as it's expected, due to its roughness, porosity and other irregularities can be described using a mathematical apparatus developed for fractal structures.

Laccase outflow from the electrolyte phase occurs only due to the departure of adsorbed molecules and can be described by the model of ideal mixing, i.e. there are no concentration gradients in this region, and the loss of the enzyme occurs only due to the adsorption:

$$V_l \frac{dc_l}{dt} = -aW_{ads} \quad (1)$$

where V_l, m^3 – volume of solution from adsorption is carried out; $c_l, \text{mol}\cdot\text{m}^{-3}$ – enzyme concentration in the solution; a, m^2 – surface area of CM, available for the enzyme adsorption; $W_{ads}, \text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, – rate of the adsorption process, expressed as in [23]:

$$W_{ads} = pJ_{ads} = pc_l \left(\frac{k_B T}{2\pi m} \right)^{1/2} \quad (2)$$

where p – adsorption probability; $J_{ads}, \text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ – adsorbate flow to the surface; $k_B, \text{J/K}$ – Boltzmann constant; T, K – temperature; m, kg – mass of adsorbate molecule.

Equation (1) was solved analytically. The expression to calculate adsorbate concentration in the solution vs adsorption time has the following form:

$$c_l(t) = c_l^0 \exp \left[-ap \left(\frac{k_B T}{2\pi m} \right)^{1/2} V_s^{-1} t \right] \quad (3)$$

where c_l^0 – the concentration of laccase in the solution at the initial time. The determination of the surface area available for adsorption is based on known data of the material specific surface area measured by Brunauer–Emmett–Teller (BET) method $S_{\text{BET}}, \text{m}^2\cdot\text{g}^{-1}$ and CM loading m_{CM}, g :

$$a = S_{\text{BET}} m_{\text{CM}}. \quad (4)$$

The adsorption probability p is fitted parameter, its value was found from the conditions of calculated data correspondence to the experimental one.

3.2. Model of laccase diffusion in carbon material

In order to carry out further calculations of oxygen bioelectrocatalytic reducing process it is necessary to obtain the information of enzyme distribution over the CM layer thickness. At modeling the process of impregnation of CM by enzyme, it was considered that the loss of substance from the local volume of the material occurs only due to its diffusion. The diffusion equation is based on the second Fick's law and contains fractional derivatives instead of the usual ones due to the fractal structure of the porous CM, the fractional derivative order γ characterizes the porosity of the material and corresponds to the fraction of the channels available to the flow, $0 < \gamma < 1$.

As it has been shown [24] in such structures ultra-slow transfer processes can be realized:

$$\frac{\partial^\gamma c}{\partial t^\gamma} = D_\gamma \frac{\partial^2 c}{\partial x^2} \quad (5)$$

where c , $\text{mol} \cdot \text{m}^{-3}$ – enzyme concentration in CM; x , m – the coordinate of active layer thickness; D_γ , $\text{m}^2 \cdot \text{s}^{-\gamma}$ – the coefficient of enzyme diffusion in a porous medium, is determined by the relation:

$$D_\gamma = D^* S^{(1-\gamma)} \quad (6)$$

where D , $\text{m}^2 \cdot \text{s}^{-1}$ – the coefficient of enzyme diffusion; S , m^2 – the cross-sectional area of the channel available for the flow, calculated from the data of the pore diameter d_p , m .

The value of the fraction of the channels available for the flow was determined from the ratio of the surface area free for the adsorption (S_{external}) to the total surface area of the material determined by the BET method (S_{BET}):

$$\gamma = S_{\text{external}} S_{\text{BET}}^{-1} \quad (7)$$

The equation of enzyme distribution over the active layer thickness (5) was solved by the finite-difference method using the implicit scheme (8) [24]:

$$\frac{c_j^{n+1} - \gamma c_j^n}{\Gamma(1-\gamma)(1-\gamma)\Delta t^\gamma} = D_\gamma \frac{c_{j+1}^{n+1} - 2c_j^{n+1} + c_{j-1}^{n+1}}{\Delta x^2} \quad (8)$$

where Γ – gamma function, n – time step, j – coordinate step.

The scheme (8) is absolutely stable and approximates equation (5) with $(2-\gamma)$ time order and 2-th coordinate order [24]. The approximation time order $(2-\gamma)$ shows that the fractional derivative equation describes the mass transfer process more accurately than the partial differential equation, where the given order is 1.

The implicit scheme (8) was solved by the numerical method using following boundary conditions: the concentration of the adsorbed enzyme at the left border: solution/CM is equal to the laccase concentration in the solution:

$$c(x=\delta, t) = c(t) \quad (9)$$

The absence of a concentration gradient along the thickness at the right boundary:

$$D_\gamma \frac{\partial c(x=h, t)}{\partial x} = 0 \quad (10)$$

The solution of equation (5) was carried out using the "floating" coordinate x in order to determine the time-varying adsorption thickness H , m . The thickness H was determined from the condition of the material balance: the mass of the enzyme left the solution is equal to the mass adsorbed in the CM.

As a result the mathematical model allows to obtain the dependence of the enzyme concentration in the solution on the time of adsorption, the values of the impregnation thickness of LM with laccase, the distribution of the enzyme concentration vs CM layer thickness and time.

4. Results and discussion

This study was carried out to establish the dependence of the laccase-based electrode activity on the catalytic layer thickness and the type of carbon material. Figure 3 shows that the adsorption values for the two materials (XC-72 and CNT) are approximately the same, but its efficiency decreases with increasing thickness of the catalytic layer for the two types of CM. Apparently, this is due to the limitations of access of the enzyme to the deeper layers of the CM. In addition, figure 3 shows the data calculated with the model equation. One can see a good agreement between calculated (lines) and experimental (dots) data.

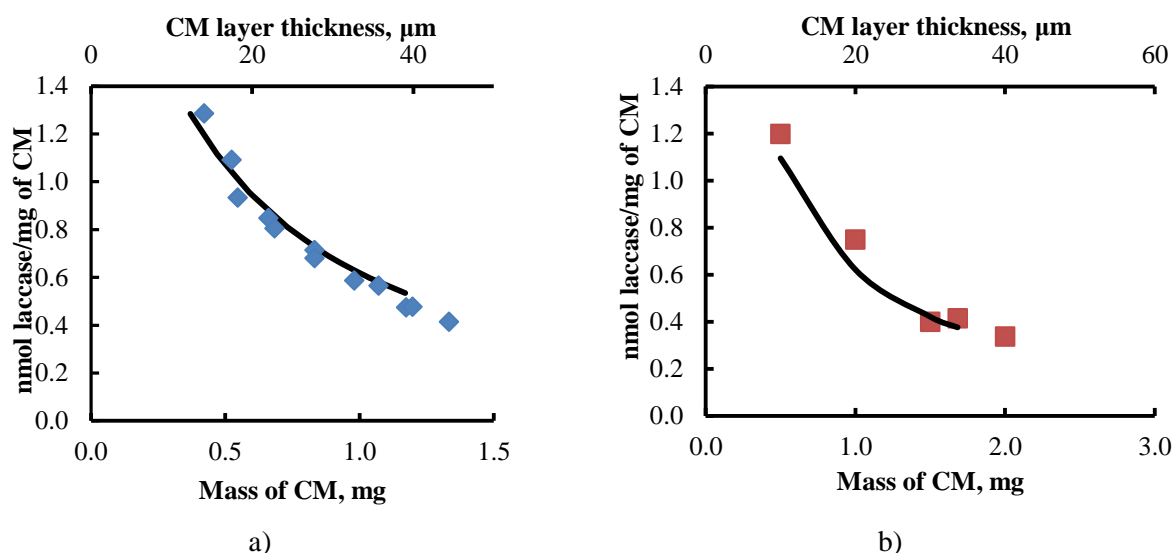


Figure 3. Dependence of the efficiency of laccase adsorption on the mass (thickness) of the active layer for: a) CNT; b) XC-72.

However, the specific activity of bioelectrodes based on laccase, adsorbed on CM of two types (figure 4), varies considerably. For carbon nanotubes, it sharply increases with increasing mass (thickness) of the CM layer to 1 mg, and then begins to decrease gradually. Obviously, only part of the adsorbed on the CM enzyme takes part in current-forming reactions, and the fraction of laccase molecules adsorbed in the electrochemically active state on nanotubes is much larger in comparison with carbon black. Probably, the enzyme, due to its size, does not penetrate deep into the CM layer but adsorbs only on the accessible surface.

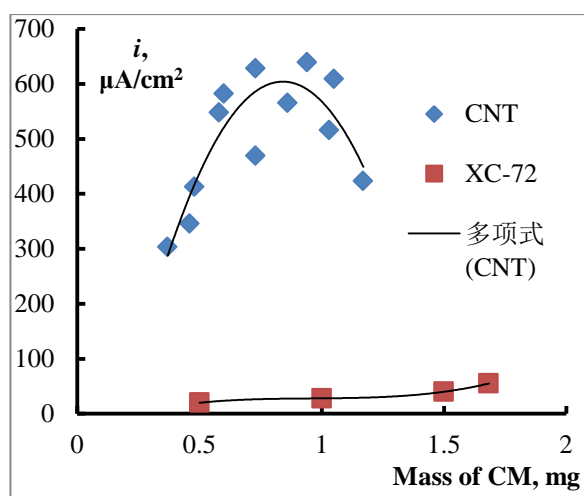


Figure 4. Dependence of the specific activity of the electrode on the mass (thickness) of the active layer (at 0.4 V, sweep rate 1 mV/s; oxygen).

Table 1. The structural characteristics of the materials

Parameter	XC-72	CNT
S_{BET} , m^2/g	230	216
V_{pores} , cm^3/g	2	3.8
S_{external} , m^2/g	150	198

Figure 5 shows photomicrographs made in the centre of collective use of the D. Mendeleev University of Chemical Technology of Russia, which depicts electrodes with CM on its surface. The photos clearly show mesopores of materials (pores between agglomerates of nanotubes or particles of carbon black). Obviously, the mesoporosity of CNTs is higher than carbon blacks, which corresponds to the data on the structural characteristics of the materials presented in table 1.

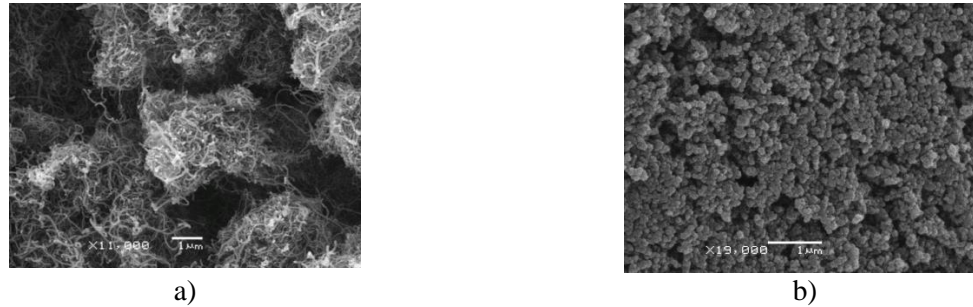


Figure 5. Photomicrographs of electrodes surface with CM on it: a) CNT, b) carbon black XC-72

Possibly, the mesoporosity of the material allows the enzyme to penetrate deep into the layer, surrounding it and increasing the possibility of its attachment by the binding centre of the second substrate responsible for the transfer of electrons from the electrode surface to the active centers of laccase. Figure 5 shows the carbon blacks maximum mesopore size is much smaller than the size of mesopores of nanotubes, so the enzyme is forced to adsorb in the surface layer of the material, where the probability of attachment in a favourable position for direct electron transfer is much smaller.

In the result of simulation following dependences were obtained: the distribution of the enzyme concentration on CM layer thickness at different process times (figure 6). As shown in figure 6, the penetration depth of the enzyme increases with time, and its concentration in the CM impregnated layers decreases insignificantly. Obtained data will be used at the next stage of mathematical modeling to estimate the dependencies of oxygen bioelectrocatalytic reduction by laccase.

Input, measured and calculated parameters are listed in table 2.

Table 2. List of parameters values used in the simulation

Parameter	Symbol	Value	Reference
Laccase diffusion coefficient	$D, \text{m}^2 \text{s}^{-1}$	1.49×10^{-10}	[25]
Temperature	T, K	298	Experimental
Volume of solution	V_l, m^3	5×10^{-7}	Experimental
CM mass on electrode	m_{CM}, kg	62×10^{-6}	Experimental
Enzyme initial concentration in solution	$c_l^0, \text{kg m}^{-3}$	0.154	Experimental
Average CNT pore diameter	$d_{\text{pore}}, \text{m}$	11×10^{-7}	[12]
Fraction of channels available to electrolyte flow	γ	0.94	Calculated (Eq. 7)
Laccase diffusion coefficient in active layer	$D_p, \text{m}^2 \text{s}^{-1}$	6.59×10^{-11}	Calculated (Eq. 6)
Adsorption probability	p	3.4×10^{-10}	Fitted

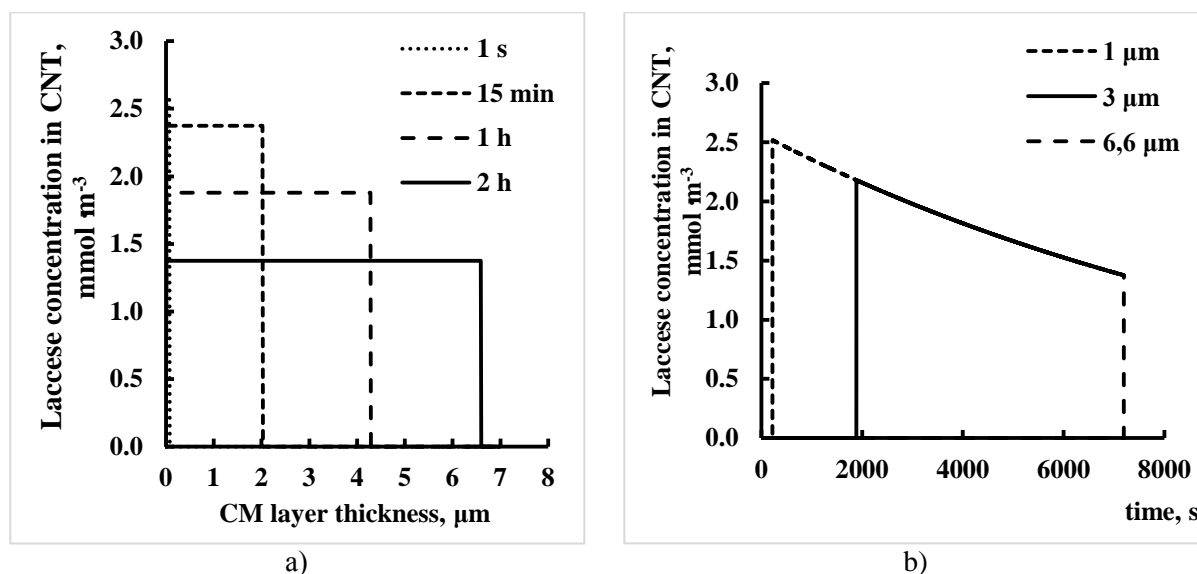


Figure 6. Distribution of the concentration of adsorbed laccases: a) vs CM layer thickness; b) vs adsorption time.

5. Conclusions

We have carried out a series of experimental studies of establishing the main dependencies of the values of laccase adsorption on carbon materials from their structural characteristics. We have developed a mathematical model of adsorption process, which predicts the amount of adsorbed enzyme on CM of different dispersity, as well as enzyme distribution over the thickness of the CM layer. The model includes the equations for enzyme concentration changing in the solution as a result of its adsorption on the surface of the CM and due to the enzyme diffusion within the thickness of the CM. We have developed an algorithm for calculating equations of the mathematical model. As a result, the dependencies of the distribution of the enzyme amount over the thickness of the CM layer, the accumulation of the substance by different layers of CM as a function of the time of adsorption, were obtained. The parameters of the laccase adsorption process at CMs were determined. Calculations for the amount of adsorbed at CM laccase for different amounts of CM and different concentrations of the initial enzyme solution are in good agreement with the experimental data. The next stage of this scientific research is the development of a mathematical model of direct electron transfer bioelectrocatalysis, which will use the obtained in this paper data and which can predict the electrochemical characteristics of a laccase-based electrode.

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