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Fractal and percolation properties of active layer structure at oxygen electrode based on nanocomposite material of dispersed carbon carrier/laccase

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Abstract

The properties of the oxygen electrode active layer with laccase immobilized on highly dispersed colloid graphite (HCG) or carbon black AD 100 are studied. It was assumed that such a composite material provides the optimum conditions for bioelectrocatalytic oxygen reduction and allows enhancing the measured current values referred to the electrode geometric surface. These expectations were not wholly justified: the employment of HCG as a carrier allowed enhancing the specific activity per laccase molecule up to 29.8 μA/pmol of the enzyme, which is five times higher than the specific activity of laccase applied to AD 100 (5.9 μA/pmol of the enzyme); however no success was achieved in forming the active layers of any considerable thickness both in the first and in the second case, as the activity decreased drastically at the increase of the layer thickness. The nature of these problems can be caused by a number of reasons: the physico–chemical and structural characteristics of the carbon materials determining, in their turn, the adsorption value and the orientation of the enzyme molecules towards the carrier and the regularities of the active layer structure consisting of the fractal clusters of carbon particles. With the help of computer modeling, the fractal and percolation properties of the active layer structure are studied. The models of the active layer structure are suggested, in which carbon particles form agglomerates of fractal cluster type. This allows to suggest a number of considerations that can help to explain the fractal and percolation effects and the effect of the carbon carrier particle size on the immobilized enzyme coverage.

Keywords: Laccase; Nanocomposite material; Active layer; Percolation; Fractal cluster

1. Introduction

One of the key problems in the construction of the biofuel cells (BFC) is the development of the enzyme-containing composite nanostructured catalysts providing the effective enzyme functioning and, correspondingly, the high and stable electrochemical characteristics. Such enzymes as laccase [1,2] and cytochromoxidase [3,4] can be used as electrocatalysts of oxygen reduction. In paper [5] BFC is suggested, in which cytochromoxidase enzyme was employed for oxygen reduction and glucose oxidase enzyme was used for glucose oxidation. The maximum capacity of such BCG constituted 4 μW cm^{-2}. In papers [6,7] BFC are considered possessing the cathode based on laccase and anode based on glucose oxidase "wired" to the electrode surface by means of a polymer modified by Os-containing complex. The capacity of BFC described in [6] constituted 150 μW cm^{-2} at the voltage of 0.55 V and the temperature of 37 °C. In the second case [7], due to the using for immobilization a polymer that is capable of high-rate electron exchange with the active center of the enzyme at the potential (of −0.19 V versus Ag/AgCl electrode), which is close to the redox potential of glucose oxidase, the voltage of a BFC constituted 0.78 V in a citrate buffer solution at pH 5.5 and 37 °C. The capacity of this BFC achieves 268 μW cm^{-2}. The hydrogen–oxygen BFC containing laccase (oxygen electrode) and hydrogenase (hydrogen elec-

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trode) enzymes is suggested in paper [8]. Its capacity is about 320 $\mu W\,cm^{-2}$.

As was shown earlier [9,10], laccase that is immobilized by adsorption at various carbon materials (carbon blacks, activated charcoals, pyrographite) provides the mediatorless (direct bioelectrocatalysis) oxygen electroreduction in weakly acidic solutions at the potentials close to the equilibrium potential of O_2/H_2O system. On the basis of the obtained data, a conclusion is made that only an inconsiderable part (~10%) of the immobilized enzyme molecules takes part in the bioelectrocatalytic reaction [2]. A supposition is made that this is due to the orientation of the adsorbed laccase molecule in respect to the electroconducting carbon material surface, which is unfavorable for the electron transfer.

The enzyme-based composite materials differ greatly from the electrocatalysts of metallic nature: firstly, enzymes feature no electronic conductivity; secondly, the bioelectrocatalyst size is comparable to the sizes of the other structural units of the composite material. Therefore, in the process of the formation of nanostructured composite material, one has to take into account the nature of the carbon carrier (dispersity, type and amount of the functional groups at the carbon material surface). This allows to provide the maximum coverage and favorable spatial orientation of enzyme molecules in respect to the carbon carrier. In this connection, in the present paper we studied the effect of the carbon material dispersity on the electrocatalytic activity of the adsorption-immobilized laccase and the effect the active layer thickness on the electrochemical parameters of the cathode. For the interpretation of the experimental results, the computer modeling of the active layer structure is performed, its percolation and fractal properties are studied, and the amount of enzyme molecules is estimated that can be adsorbed at the carrier.

2. Experimental methods and estimation of characteristic lengths

In the present work, laccase of *Coriolus versicolor* extracted according to the method [11], pyrochatecol (Aldrich) additionally purified by sublimation, citric acid, and sodium hydrophosphate ($Na_2HPO_2{\cdot}12H_2O$) (Mir Reaktivov) were used. The carbon materials were: acetylene carbon black AD 100 with the specific surface of 100 $m^2\,g^{-1}$, and highly dispersed colloid graphite (HCG) with the specific surface of 2000 $m^2\,g^{-1}$, obtained from Elektrougli (Russia).

The measurements were performed in a three-electrode glass cell with separated electrode compartments. The working electrode was a floating electrode [12], to which the composite carbon laccase-containing material was applied. The floating electrode is a tablet obtained by pressing (the pressure constituted 5.5 MPa cm^{-2}) 300 mg of hydrophobized carbon black containing 35 wt.% of fluoroplast (Teflon). The tablet possesses the thickness of 0.3 cm and the area of the surface contacting the electrolyte is 1 cm^2. The electrode of such construction is a model of a gas-diffusion cathode and provides the equal accessibility of the active layer in respect to oxygen supply. The reference electrode is the saturated silver–silver chloride electrode; all the potential values are presented versus this electrode. The electrolyte was 0.15 M citrate–phosphate buffer solution with pH 4.0 (prepared from 0.15 M solutions of citric acid and sodium hydrophosphate). The measurements were performed at the room temperature in the atmosphere of oxygen, air, or argon.

The enzymatic activity of laccase was determined spectrophotometrically from the rate of pyrochatechol oxidation [13]. The enzymatic activity unit was the amount of laccase that oxidizes 1 μmol of pyrochatechol per min. The spectrophotometric measurements were performed in a cuvette with the thickness of 2 cm. 5 ml of buffer solution with pH 4.0 containing 5.5 mg of pyrocatechol and 3.5 μg (58 pmol) of laccase were placed into the cuvette. The optical density of the solution was registered at the wavelength $\lambda = 375$ nm ($\varepsilon = 36.0\,mM^{-1}\,cm^{-1}$) [14]. The activity of laccase determined by means of this method per 1 mg of the enzyme constituted 30 U mg^{-1}.

To prepare the nanocomposite material based on laccase and polydispersed carbon material (HCG, AD 100), 2 mg of carbon material were placed into 100 μl of citrate–phosphate buffer solution of laccase containing 16 μg of laccase; the mixture was thoroughly mixed and conditioned for 2 h. Then a sample of the mixture was applied on the floating electrode and dried at the room temperature.

The amount of strongly adsorbed enzyme at the carbon material was determined by means of spectrophotometry. For this purpose, the floating electrode with applied active layer was placed at the surface of the citrate–phosphate buffer solution with the volume of 10 ml, and in 2 h time the amount of the enzyme that passed into the solution was determined by its activity, using the method described above. The amount of strongly adsorbed enzyme was determined as the difference between the amount of enzyme applied and enzyme in the solution.

The electrochemical measurements were performed by means of the cyclic voltammetry (CVA) method using potentiostat IPC-05 made in the Institute of Physical Chemistry, RAS.

The polarization capacitance of the electrodes was calculated from CVAs measured in the potential range of 0.3–0.5 V in the solutions deaerated by argon. It was shown in the preliminary experiments that the amount of electricity calculated in the given potential range was independent of the potential sweep rate up to $\nu = 5 \times 10^{-4}\,V\,s^{-1}$ if the amount of carbon material or composite at the electrode did not exceed 3 mg in the case of HCG and 5 mg in the case of AD 100. In this connection, all the calculations were performed with the data obtained at CVA potential sweep rate of $1 \times 10^{-4}\,V\,s^{-1}$.

To determine the diffusion and ohmic resistances due to the limitations of oxygen diffusion in the composite layer and the ohmic resistance of the electrolyte, the values of the characteristic ohmic and diffusion process lengths were calculated. The characteristic diffusion process length was

determined according to the following formula [15]:

$$L_{\mathrm{dif}} = \left(\frac{nFD^{*}c_0}{\check{N}_{\mathrm{A}} J_0}\right)^{1/2} \quad (1)$$

where n is the amount of electrons taking part in the reaction, F is Faraday's number; D^{*} is the effective diffusion coefficient, c_0 is the gas solubility, $\check{N}_{\mathrm{A}}$ is the amount of enzyme molecules in a volume unit of the active layer that take part in the electrochemical reaction, J_0 is the current generated by enzyme molecule in the absence of the diffusion limitations.

To calculate the characteristic ohmic length, the following expression was used:

$$L_{\mathrm{ohm}} = \left(\frac{RTk^{*}}{F\check{N}_{\mathrm{A}} J_0}\right)^{1/2} \quad (2)$$

where R is the gas constant, T is the temperature, k^{*} is the effective specific ionic conductivity. Using expressions (1) and (2), we obtain:

$$\left(\frac{L_{\mathrm{dif}}}{L_{\mathrm{ohm}}}\right) = \left(\frac{nF^2Dc_0}{RTk}\right)^{1/2} \quad (3)$$

In the calculations, it was assumed that the amount of electrons $n=4$, $D=D^{*}g_{\mathrm{liq}}=6.0\times10^{-6}\,\mathrm{cm^2\,s^{-1}}$ (g_{liq} is the liquid porosity of the composite equal to 0.5), $c_0=9.5\times10^{-7}\,\mathrm{g\,mol\,cm^{-3}}$, $k=k^{*}g_{\mathrm{liq}}=2.4\times10^{-2}\,\mathrm{Ohm^{-1}\,cm^{-1}}$, $T=298\,\mathrm{K}$. $L_{\mathrm{dif}}/L_{\mathrm{ohm}}=6\times10^{-2}$. As L_{ohm} is higher than L_{dif}, further we will account only for the diffusion limitations.

3. Results and discussion

The studied dispersed carbon materials, HCG and AD 100, contain the particles with different sizes. At Fig. 1, the structural elements of the composite materials based on laccase, HCG, and AD 100 and their sizes are shown. The average diameter of HCG particles constitutes 3–4 nm, which is comparable with the diameter of the laccase molecule (~5 nm). The size of carbon black particles is 10 times higher and constitutes 30–50 nm. Besides, the carbon black particles usually form agglomerates [16].

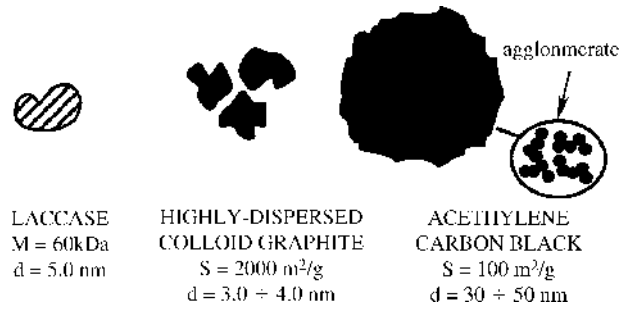

Fig. 1. The structural elements of the composite material based on: (a) laccase ($M=60$ kDa, $d=5$ nm); (b) HCG ($S=2000\,\mathrm{m^2g^{-1}}$, $d=3$–4 nm); and (c) AD100 ($S=100\,\mathrm{m^2\,g^{-1}}$, $d=30$–50 nm).

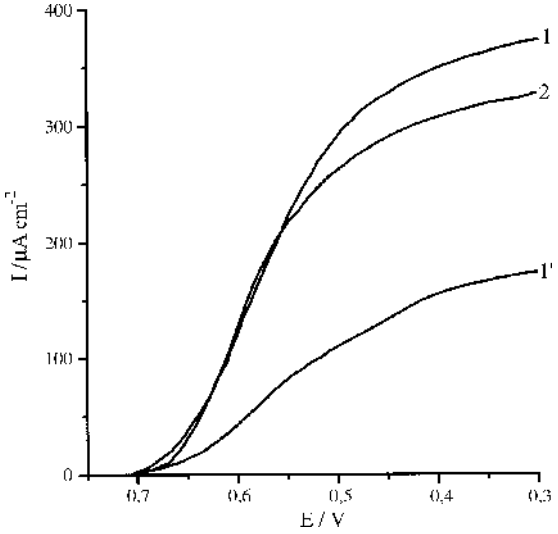

Fig. 2. Polarization curves of oxygen reduction by laccase immobilized at: 1, 1′-HCG and 2-AD 100. The curves were measured in the atmosphere of: 1,2-oxygen, 1′-air. The amount of composite material is 0.5 mg cm^{-2}. The potential sweep rate is 0.1 mV s^{-1}.

In the oxygen atmosphere, both in the case of the active layer based on laccase and HCG and that based on laccase and AD 100, the steady-state potentials are established that are rather close to each other (0.705 and 0.700 V, correspondingly) and the equilibrium potential of oxygen reaction (0.770 V). At the cathodic polarization of 0.250–0.400 V from the steady-state potential, a plateau is observed at the polarization curves (Fig. 2) that corresponds, as shown earlier [2], to the limiting kinetic current. At the polarization curves presented in Tafel coordinates one can distinguish the parts with the slopes of 0.015, 0.030, 0.060, and 0.120 V, which agrees with the slope values obtained earlier at the smooth [1,2] and dispersed [10] carbon materials with adsorbed laccase. This points to the fact that the mechanism of oxygen electroreduction reaction does not alter in the cases of laccase immobilization at various carbon materials. In air, when using HCG-based composite, the values of the limiting kinetic current of oxygen reduction are two times lower than those obtained in the oxygen atmosphere (Fig. 2, curves 1, 1′).

In Table 1 the data are presented on the amount of the strongly adsorbed enzyme at HCG and AD 100. As can be seen, the amount of the adsorbed enzyme in the case of HCG is several times lower than at the acetylene carbon black. In the case of adsorption at HCG, about 80% of laccase are desorbed into the solution bulk. Basing on these data, the values were determined of the specific currents of oxygen reduction by laccase adsorbed at HCG and acetylene carbon black differing by five times (Table 1).

Probably, such a difference in the specific current of oxygen reduction by laccase within the active layer based on HCG and AD 100 is caused by the structure of the nanocom-

Table 1
Values of laccase adsorption (Γ)[a] and electrochemical parameters of the composite materials

Carbon material	Γ (pmol mg^{-1}$_{comp}$)	E_{st} (V)	I_{max} (μA cm^{-2}) (E = 0.30 V)	j (μA pmol^{-1})
HCG	25	0.705	373	29.8
AD 100	110	0.700	326	5.9

[a] A sample of the composite mixture containing 130 pmol of laccase per 1 mg of carbon material was applied to the electrode.

posite material. As the size of HCG particles is comparable with the size of laccase molecules (Fig. 1) and the concentration of laccase molecules is two orders of magnitude lower than that of the carbon carrier particles (see the estimations below), at first a supposition was made that such an active layer structure is created, in which each enzyme globule is surrounded by HCG particles (Fig. 3a).

The process of bioelectrocatalytic oxygen reduction by laccase can be presented by the following scheme [2] that accounts for the concepts of the enzymatic catalysis [17] and includes the step of enzyme–substrate complex formation (4), the step of synchronous transfer of the first two electrons (5), the step of transfer of another two electrons and dissociation of the enzyme–substrate complex (6):

$$L{+}O_2 + H_2O \xrightarrow{k_1} LHO_2OH \quad (4)$$

$$LHO_2OH + 2e + 2H^+ \underset{k_2}{\overset{k_2}{\rightleftarrows}} L(OH)_2 + H_2O \quad (5)$$

$$L(OH)_2 + 2e + 2H^+ \xrightarrow{k_3} L{+}2H_2O \quad (6)$$

In the oxygen atmosphere, in the limiting current range, the value of catalytic constant k_{cat} of the oxygen electroreduction reaction can be compared to the value of k_{cat} obtained from the enzymatic catalysis data [18]. In the enzymatic catalysis k_{cat} is independent of the second (organic) substrate nature and its value constitutes 120–130 s^{-1}. The calculation of k_{cat} of the reaction of oxygen electroreduction by laccase was performed according to the following formula:

$$I_{max} = nFk_{cat}\Gamma A, \quad (7)$$

where I_{max} is the maximum current; A is the electrode surface area. The obtained value of k_{cat} of HCG-based composite with adsorbed laccase constitutes ~70 s^{-1}, which is comparable with k_{cat} value in the enzymatic catalysis. This provides a basis for assuming that in these conditions practically all the adsorbed enzyme molecules take part in the process of the direct bioelectrocatalytic oxygen reduction (Fig. 3a). In the case of laccase adsorption on the carbon black with the particle size of about an order of magnitude higher than that of HCG (Fig. 1), only a part of the adsorbed enzyme molecules assume the orientation towards the electroconducting carbon matrix that provides the direct electron transfer between the electrode and the active center of the enzyme (Fig. 3b). If one assumes that the activity of laccase adsorbed at carbon black is approximately equal to the activity of laccase adsorbed at HCG, then the calculation demonstrates that only a part of enzyme molecules adsorbed at carbon black (less than 20%) participate in the direct bioelectrocatalysis. Catalytic constant k_{cat} determined for the active layer based on carbon black corresponds to the value of ~15 s^{-1}.

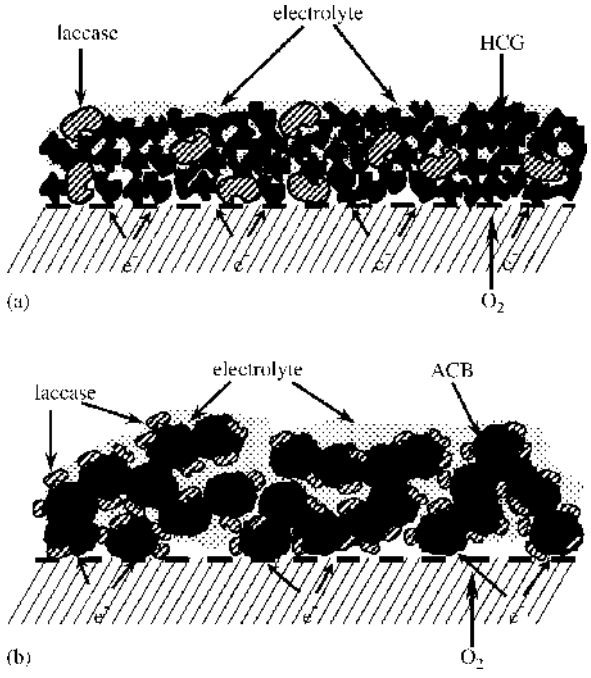

Fig. 3. The ideal structure of the active layers based on laccase immobilized at HCG (a) and AD 100 (b).

The obtained data evidence that at the increase of the amount of laccase immobilized on HCG the current value per electrode surface can be increased. The low laccase adsorption value on HCG can be caused by a number of reasons, among which there are the hydrophobic–hydrophilic interactions between the carbon carrier and enzymatic globule, electrostatic interactions, and also the effect of the ratio of the amount and size of the carrier particles and laccase molecules.

Further, the concentration of laccase molecules is estimated in the solution and in the composite. For definiteness sake, we chose HCG as the carrier. It is shown that only 25 pmol (1.5×10^{13} molecules) of the possible 130 pmol of laccase are adsorbed on 1 mg of the carbon material. According to the experimental conditions, the initial laccase concentration in 100 μl (0.1 cm^3) of the solution c_E^* constituted 2670 pmol or 1.6×10^{15} molecules cm^{-3}. The composite that is formed in the solution occupies only a part of the initial volume (less than 0.1 cm^3); therefore it is difficult to determine the exact concentration of laccase molecules in the composite bulk. Let us determine the amount of HCG particles contained within 1 mg of this material assuming

that all the graphite particles are spherical and their diameter $d_G = 3.5$ nm. Then the sought for amount of graphite particles is c_G. Carbon density $\rho = 2\ g\ cm^{-3}$. Thus, 1 mg of HCG contains 2.2×10^{16} particles (4.4×10^{17} particles cm^{-3}). Therefore, a molecule of laccase within the composite corresponds to $4.4 \times 10^{17}/1.6 \times 10^{15} = 275$ particles of HCG. It might seem that, in the case of such a ratio of HCG particles and laccase molecules within the composite, it can be assumed that the schematic distribution of the particles in the active layer presented at Fig. 3a is correct and therefore it can be considered that laccase molecules are surrounded on all the sides by HCG particles. However, this opinion disagrees with the experimental data presented below.

The active layer thickness is of great importance in the case of the construction of the gas-diffusion electrode with practically acceptable overall current densities. In this connection, the effect of this parameter on the electrochemical characteristics was studied in the reaction of the oxygen reduction at the composite electrodes of two types. It was shown that the effect of the catalyst amount applied to the electrode on the specific activity value remains the same both in the potential range close to the steady-state potential and in the case of the polarization of above 0.1 V (Fig. 4), but it is different for various carbon carriers. For the composite based on HCG, a weak dependence is observed of the specific current of O_2 reduction by laccase at the composite amount from 0.3 up to 0.5 mg cm^{-2}; at the increase of the active mass amount at the electrode up to 1.5 mg cm^{-2} the specific current of oxygen reduction decreases by more than three times (Fig. 4a). In the case of the composite based on carbon black, at the increase of the amount of the composite material at the electrode from 0.5 up to 2.0 mg cm^{-2} the value of the specific current of oxygen reduction decreases by 1.5–2 times, and the further increase of the active layer thickness produces practically no effect on the specific current value (Fig. 4b).

To elucidate the nature of the abrupt catalyst activity decrease at the increase of the active layer thickness, the values of the characteristic diffusion lengths were calculated. For the HCG-based active layer at the potential of 0.30 V ($I = 373\ \mu A\ cm^{-2}$, layer thickness $\Delta = 5\ \mu m$), we obtain the diffusion length equal to 12.1 μm, which is lower than 10 $\Delta = 50\ \mu m$. This points to the fact that at the high cathodic polarizations the electrochemical process of oxygen reduction is limited by the gas supply in the reaction zone. At the low polarizations ($E = 0.68$ V), in the case of the HCG-based active layer, the value of the reduction current is 21.3 μA cm^{-2} and the obtained diffusion length corresponds to the value of 50.7 μm, which is comparable with 10 Δ. Consequently, at the potentials close to the steady-state value, the process of oxygen reduction by laccase occurs in the kinetic mode. Herewith, the initial parts of the polarization curves of oxygen reduction at the composites based on HCG and AD 100 coincide, and at the potentials that are more cathodic than 0.55 V the curves diverge (Fig. 2, curves 1, 2). As the active layer thickness grows (Fig. 4), for both carbon materials the potential range, in which the electrochemical process

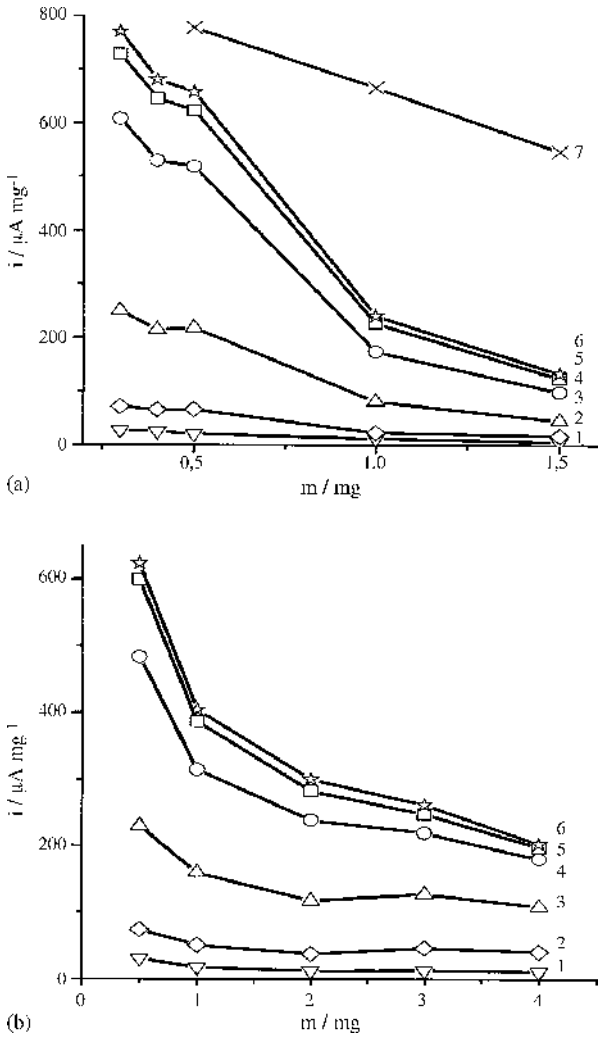

Fig. 4. Dependence of the specific electrode activity on the active layer mass (thickness) at the potentials of, *V*: 1–0.68, 2–0.65, 3–0.60, 4–0.50, 5–0.40, 6 and 7 (theoretical curve)–0.30. The rate of potential sweep is 0.1 mV s^{-1}. The atmosphere is oxygen. (a) laccase adsorbed at HCG and (b) laccase adsorbed at AD 100.

is limited by kinetics, decreases, while the potential range, in which the diffusion limitations affect the process of oxygen reduction, grows.

The values of the characteristic diffusion length and characteristic diffusion current determined from the experimental data allow calculating the current values for various active layer thicknesses. Relating the obtained current values to 1 mg of the active mass, we obtain the theoretical curve (Fig. 4a, curve 7), which accounts for the diffusion limitations. Let us note that the experimental curve (6) lies sufficiently below the theoretical curve (7). This means that account for the diffusion limitations only is insufficient for correlating the theory and the experiment. Probably, there exists an additional factor that affects the electrochemical activity of the composite at the increase of its thickness. Such a factor, e.g., can be the percolation effect [19], caused by the distortion of the electronic contact between the carbon par-

ticles within the composite material. This means that Fig. 3 represents an idealized concept of the composite. If the composite structure with HCG and carbon black AD 100 shown at Fig. 3 had been realistic, there would have been no percolation effect.

4. Computer modelling

Thus, there are two facts that require explanation: 1) the reason for the lower carrier surface coverage (especially HCG) by enzyme is unclear; 2) the nature of percolation effects observed at the increase of the electrode thickness is unclear. We suppose that the correct interpretation of the experiments can be provided when one takes into account that the carbon carrier particles in the solution aggregate into a special kind of structures: the random fractal clusters.

The problem of obtaining nanocomposites based on carbon carriers and enzymes of perfect structure is very complicated, as three processes can occur simultaneously: the interaction between the carbon carrier particles, the interaction between the enzyme molecules (it is known that protein molecules in aqueous solutions tend to form agglomerates), and the interaction between the carrier particles and enzyme molecules. To provide the directional interaction between the enzyme molecules and carbon carrier, of great importance are: the concentration on composition of the solution, employment of surface active agents, hydrophobic–hydrophilic interactions. To decrease the free energy of the whole system, it is expedient to decrease the overall hydrophobicity. Probably, such a phenomenon was observed when laccase molecules possessing both the hydrophobic and hydrophilic parts at the globule surface were mixed in the solution with carbon black (AD 100). Probably for this reason the amount of strongly adsorbed laccase molecules at HCG (hydrophilic material) turned out to be 4.5 times less than in the case of AD 100 (partly hydrophobic material).

The basis of our concept on the aggregation of lyophobic particles in the solutions during the two recent decades was mainly obtained due to the employment of the theory of fractals [20] and computer modeling methods. For the sake of simplicity and vividness, we studied not the three-dimensional, but two-dimensional objects, and all the calculations were performed for the system of HCG + laccase. The conclusions that follow from the computer modeling results presented below are provisional. At present, one can speak only about the qualitative interpretation of the experimental effects that we observed because a number of the computer model parameters, such as the form and mutual aggregation ability of carbon particles and laccase molecules, were chosen arbitrarily. Undoubtedly, the transition to 3D-computer models requires additional study of the composite structure using micrographic imaging technique, such as transmission electron microscopy etc. Therefore, we chose the computer modeling mainly to pay serious attention to the necessity of accounting for the fractal and percolation properties of the studied composite materials.

Let us assume that the active layer is divided into square cells with conventional size of $L \times L$, and the fractal clusters are placed into these cells. There is a great number of computer models describing the growth of fractal clusters [21]. We chose the simplest and most often employed approach suggested in [22]: diffusion limited aggregation (DLA model). We assume that the particle movement occurs at the square lattice of sites, that the probability of aggregation is equal to 1 (a single contact is sufficient for the aggregation of two objects in the solution), and that the particles participate in Brown movement. In this case, the limiting step is obviously the diffusion transport of the particles towards the cluster.

The bulk concentration of HCG particles in the solution for laccase immobilization, as follows from the experimental method, is considerably higher than the enzyme concentration. In this connection, one can assume that the enzyme molecules due to their very low concentration feature practically no interaction, and HCG particles due to their large amount and high mobility mainly succeed in the achieving the interaction between themselves forming fractal clusters. Then laccase molecules are fastened onto such a structure formed in the solution. Therefore, the main space after the drying of the sample is filled with HCG agglomerates. When solving the problem as to the occurrence of the immobilization act at the contact between the enzyme molecule and the fractal cluster, let us assume that the immobilization occurs only in the case when the enzyme features a high enough contact surface with the cluster-forming carrier particles. Thus, there are two parameters: the relative size of the enzyme molecules and the minimum contact surface needed for the fastening of the enzyme molecule at a random fractal cluster.

The modeling of the process of enzyme molecule landing on the perimeter of the fractal center formed by carbon particles is shown at Fig. 5. The analysis of Fig. 5 demonstrates that the molecules that were attached first often hinder the other enzyme molecules to penetrate the inner regions of the fractal cluster. Therefore, not all the places at the cluster perimeter are accessible for the enzyme molecules.

Let us vary the sizes of enzyme molecules and the conditions, at which the enzyme molecule contacting the fractal cluster can be considered immobilized. Fig. 6a and c imply that the immobilization requires the contact of one of the enzyme molecule sides with two (Fig. 6a) or three (Fig. 6c) carrier particles. Unlike the situation described at Fig. 5, where the enzyme immobilization occurs at the contact with any of the carrier particles of the cluster, Fig. 6b and d imply that the formation of a strong bond between the enzyme molecule and cluster requires the contact between the whole surface of two neighboring sides of the enzyme molecule with, correspondingly, four (Fig. 6b) or six (Fig. 6d) carrier particles. Comparing all the parts of Fig. 6, we see that at the same random fractal cluster with the size of 21×21, depending on the landing conditions of the enzyme molecules that

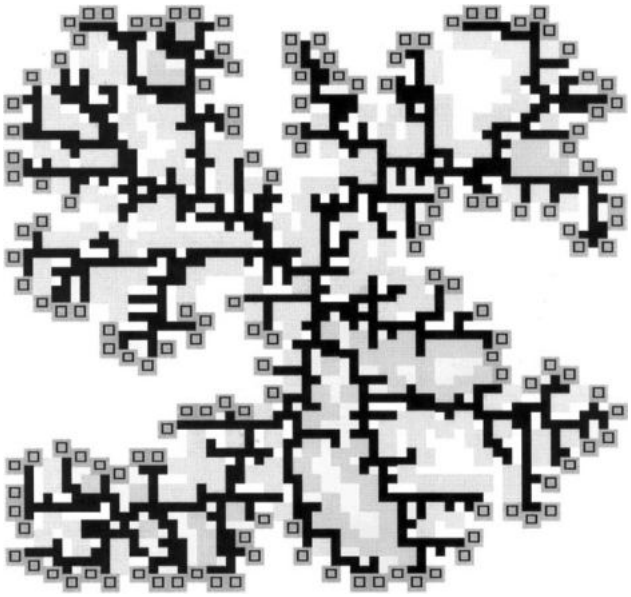

Fig. 5. Distribution of enzyme molecules (a square of conventional size of 1×1 designated by gray squares with a black frame inside) at a random fractal cluster of carrier particles (a square with the size of 2×2 marked black) with the size of 61×61. All the sites at the cluster perimeter that can potentially be filled by the enzyme molecules are marked gray. The amount of the carrier particles is 908, the number of the enzyme molecules immobilized at the cluster is 146, the number of all the sites at the cluster perimeter accessible for the enzyme molecules is 374. The fractal dimension of the cluster $D = 1.6$.

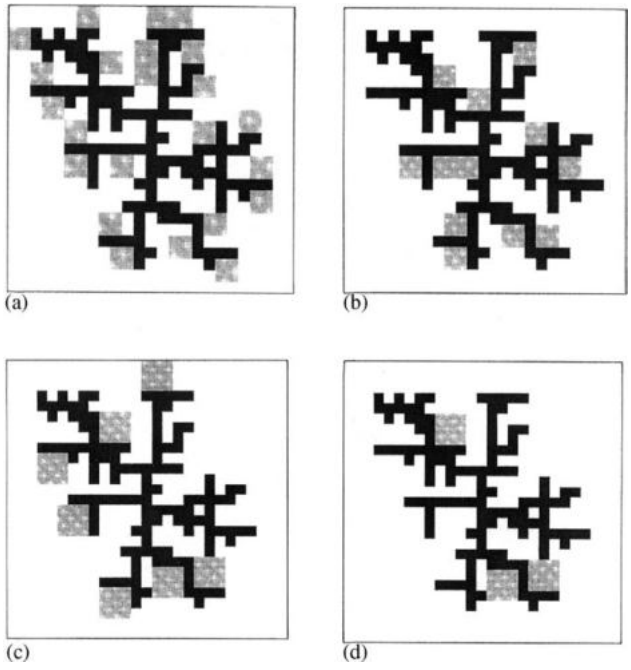

Fig. 6. Demonstration of how the number of immobilized enzyme molecules (marked gray) from a fractal cluster of carrier particles (size 21×21, the cluster is marked black; it consists of the particles with the conventional size 1×1) is changed, as dependent on the size of the enzyme molecules (size $p \times p$) and conditions of their attachment to the cluster perimeter (variant 1: by a single side of the enzyme molecule; variant 2: by two neighboring sides of the enzyme molecule). *a*–$p = 2$, variant 1, *b*–$p = 2$, variant 2, *c*–$p = 3$, variant 1, *d*–$p = 3$, variant 2.

Table 2
The main parameters characterizing the process of enzyme immobilization at a random fractal cluster L fractal cluster size; w_E the amount of immobilized molecules, $w_E{}^*$ the amount of sites at the fractal cluster perimeter that can potentially be filled by enzyme molecules; c_E the density of the number of immobilized molecules at the fractal cluster

L	m	w_E	$w_E{}^*$	c_E
–	1	23	31	0.052
21	2	7	8	0.016
–	3	3	4	6.8×10^{-3}
–	1	255	387	0.039
81	2	80	125	0.012
–	3	16	26	2.44×10^{-3}
–	1	1296	1814	0.032
201	2	402	560	9.95×10^{-3}
–	3	66	98	1.63×10^{-3}

gradually become more complicated, the amount of attached enzyme molecules at the cluster decreases and at the transition from Fig. 6a and d it constitutes 23, 12, 7, and 3 molecules, correspondingly.

Here, three variants, – a, c, and d, – are chosen out of the four variants of Fig. 6 and designated by numbers m ranging from 1 to 3, correspondingly. The dependence on fractal cluster size L of the main parameters characterizing the enzyme immobilization process: the amount of immobilized molecules w_E, the amount of sites at the fractal cluster perimeter that can potentially be filled by enzyme molecules $w_E{}^*$, and the density of the number of immobilized molecules at the fractal cluster c_E is presented in Table 2. The growth of the enzyme molecule size causes the decrease of the amount of immobilized laccase molecules by about three times, and the complication of the requirements towards the immobilization act decreases the amount of immobilized enzyme molecules by yet approximately five times. Due to this, the amount of the carrier particles per one immobilized enzyme molecule grows up to 100. We also see that c_E decreases down to thousandth parts.

Let us consider the nature of the percolation effects observed at the increase of the electrode active layer thickness. The fractal, percolation effect, as we call it, consists in the aggregation of the particles of the porous carrier into random fractal clusters connected with each other with the probability that differs from unity. Therefore, the electrons needed for performing bioelectrocatalysis can penetrate the active layer to a limited depth.

The presence of the percolation limitations can be observed from the dependences of the specific capacitance on the composite mass presented at Fig. 7. In the case of HCG without laccase, the independence of the specific capacitance on the active layer thickness remains up to 0.3 mg cm^{-2}; at the further increase of the composite amount the capacitance decreases abruptly (Fig. 7, curve 1′). At the introduction of laccase into the active layer the specific capacitance gradually decreases at the increase of the active layer thickness; herewith, up to 0.5 mg cm^{-2} the specific capacitance value is lower than the specific capacitance value at pure HCG (Fig. 7, curves 1, 2). Probably, laccase that is not electron-conducting

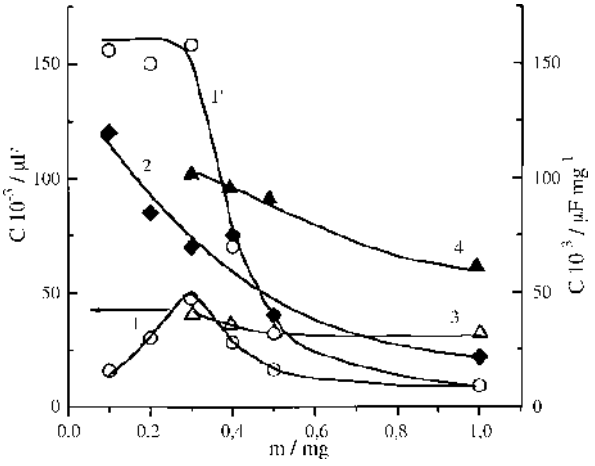

Fig. 7. Dependence of electrode capacitance (1′) and specific capacitance (1–4) on the active layer mass (thickness). 1, 1′–at the electrode of HCG; 2–at the electrode with laccase adsorbed on HCG; 3–at the electrode of AD 100; 4–at the electrode with laccase adsorbed on AD 100. The atmosphere is argon.

introduces an additional contribution into the dissipation of the fractal clusters of HCG particles. Carbon black features a different pattern. The specific capacitance at pure carbon black turned out to be two times lower than that at the composite of carbon black with adsorbed laccase (Fig. 7, curves 3, 4). The reason of this can be the hydrophilization by enzyme at its adsorption of the carbon black surface inaccessible for the electrolyte. However, both in the case of HCG and carbon black, the specific capacitance value decreases gradually at the increase of the active layer thickness.

The supposition about the percolation limitations seems rational; however, it is unclear why the electroconducting carbon particles gathering into agglomerates in the solution and later becoming the material for the composite do not provide the electron transfer in the highly thick layers. A supposition was made that the reason for the decrease of the specific activity of immobilized laccase in the reaction of oxygen reduction may consist in the distortion of the electronic contact between the carbon particle agglomerates in the composite material. It is also possible that the reason for the percolation limitations lies in the existence of very thin hydrate inter layers between the carbon material particles in the agglomerates. It seems that these hydrate (solvate) layers that hinder the electron transfer between the neighboring carbon particles in agglomerates can explain the discovered percolation phenomena. However, this is probably not so. Firstly, the composite was subjected to drying, which must have eliminated the thick liquid interlayers (∼100 nm). Secondly, the thin hydrate interlayers (∼1 nm) due to the tunneling effect [23] cannot be an obstacle for the current flow between the neighboring carbon particles. Thirdly, the percolation effects appear only at the micron composite thicknesses, while the hydrate bridges, if playing the decisive role, must start working much earlier, already at nanometer thickness, at the distances commensurable with the sizes of separate carbon particles.

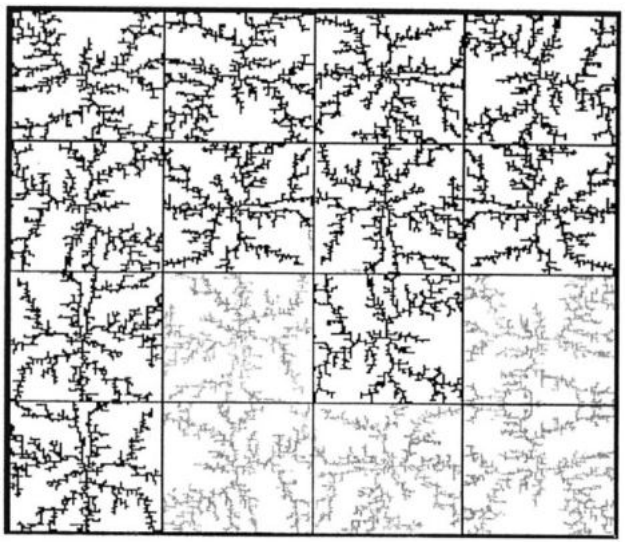

Fig. 8. The model of the porous electrode active layer. Every cell contains random fractal clusters with the size of 71 × 71. The current tap borders on the upper active layer side. The cells having access to the current tap and containing electrons are marked black; the cells that are isolated from the current tap are marked gray.

At Fig. 8, a two-dimension model of the composite is demonstrated; it is a square flat lattice of sites with the size of 4 × 4. Each square cell of this lattice holds fractal clusters the centers of which are the points in the centers of the square cells. All the cells of the upper row in immediate contact with the current tap obtain electrons. As the probability of the contact of two fractals in the neighboring cells differs from unity, in cell rows 2–4, as shown on Fig. 8, there already appear cells that are isolated from their neighbors and contain no electrons.

Further, the estimation was performed of the probability of contact between the fractal clusters in the neighboring cells of a flat square lattice γ (Fig. 9). As predicted by the percolation theory [19], in a square lattice of bonds (in our model, a

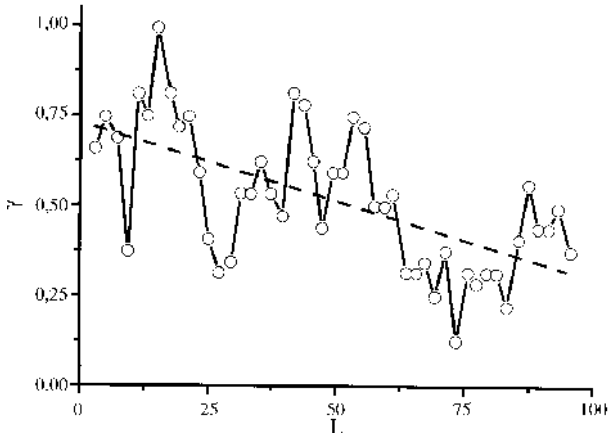

Fig. 9. Dependence of the contact probability between the two neighboring lattice cells modeling the active layer of the porous electrode (Fig. 8) on the length of the square that limits the fractal cluster. The dashed curve is the approximation of the tendency of the contact probability variation.

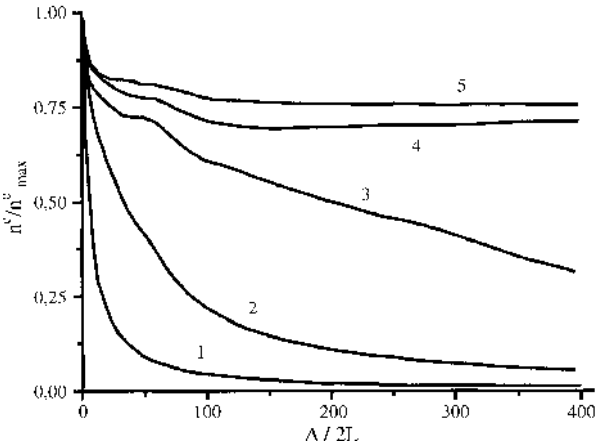

Fig. 10. Dependence of relative supply by electrons of the lattice layers (relative size of 400 × 400) on the distance between the layer and the current tap γ: 1–0.4, 2–0.475, 3–0.5, 4–0.51, 5–0.515.

"bond"s each side that is common for two contacting cells) the percolation threshold leads to the achievement of $\gamma = \gamma^* = 0.5$. At $\gamma < \gamma^*$ (curves 1, 2 of Fig. 10) the electrons can penetrate only the cell layers that are immediately contacting on the current tap (Fig. 8). And at $\gamma > \gamma^*$ (curves 4, 5 of Fig. 10) the electrons achieve any depth of the composite uniformly.

Thus, the fractal-percolation effect can explain the experimental data in the case of the non-concentrated solutions of carbon particles. The theory developed here can lead to a practical conclusion. To escape the limitations of the fractal-percolation character, one should increase the concentration of carbon particles in t he solution. This will result in the decrease of the average fractal cluster size L (Fig. 11a) and the increase of parameter γ (Fig. 12). If the concentration of carbon particles exceeds the critical value (the value of γ^* parameter is achieved), then the fractal percolation effect disappears.

5. Conclusion

The performed analysis of the experimental data using the percolation and fractal theory and computer modeling allows to conclude, that the enhancement of the structure of nanocomposite layers of HCG particles (this carrier is preferable to AD 100) and laccase and the increase of the currents at these electrodes require:

1. The elimination or decrease of the fractal-percolation limitations that hinder the increase of the active layer thickness. For this purpose, as predicted by the theory, when preparing the composite, the concentration of HCG for the enzyme immobilization in the solution must be increased.
2. The elaboration of the methods of composite preparation to increase the enzyme coverage of the carrier surface, including the choice of the optimum concentrations and sizes of the particles of carrier and enzyme.

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