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Apparent Michaelis constant of the enzyme modified porous electrode

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Plate-gap model of enzyme doped porous electrode was utilized in order to calculate apparent Michaelis constants (K_M^{app}) and apparent maximal currents (I_{max}^{app}) of modeled amperometric biosensor for the wide range of given reaction/diffusion parameters. It was found that K_M^{app} of plate-gap biosensor linearly depends on I_{max}^{app} when rates of enzymatic reaction are lower than critical. Theoretically predicted linear correlation between apparent parameters was observed experimentally for the case of carbon paste electrodes, which were modified by PQQ-dependent alcohol dehydrogenases. At overcritical rates (or apparent maximal currents), $K_M^{app}$is practically independent on Michaelis constant of soluble enzyme. Therefore, apparent Michaelis constant can be regarded as biosensor's topology representing parameter which, in fact, is not related to the specificity of enzyme kinetics. High and rate-independent values of K_M^{app} indicate that reaction proceeds at substrate-exposed top layer of the gap. In this case, reaction–diffusion system formally is stratified into separate reaction (top) and diffusion (bottom) zones. Topology of such reaction–diffusion system reminds "inverted" planar electrode, which contains diffusion layer below reaction layer. The net effect of plate-gap topology of working electrode on apparent Michaelis constant is similar to the effect of diffusion layer covering enzymatic planar electrode.

KEY WORDS: reaction–diffusion, modeling, amperometric biosensors, carbon, PQQ-dependent dehydrogenase

1. Introduction

Working electrode of enzymatic biosensor is rather complex device. It consist of the conducting material (metal, carbon, or carbon paste) coated with

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a biochemical film [1]. Despite of many efforts to model complex reaction–diffusion processes and consequent responses of such electrodes [2–7], researchers are subjected to use the simplest mathematical expression which fits their data. For example, dependences of experimentally measured steady state currents (I) on concentrations of substrates (S) of enzymatic reactions are often fitted by hypherbolic functions:

$$I = I_{\max}^{\mathrm{app}}\, S/(K_{\mathrm{M}}^{\mathrm{app}} + S), \tag{1}$$

where $I_{\max}^{\mathrm{app}}$ and $K_{\mathrm{M}}^{\mathrm{app}}$ are "apparent" parameters characterizing action of enzymatic biosensors [8]. Apparent Michaelis constant $K_{\mathrm{M}}^{\mathrm{app}}$ is a key technical characteristic of the amperometric biosensor. It represents the linearity of the calibration curve (1). On the other hand, $K_{\mathrm{M}}^{\mathrm{app}}$ qualitatively characterizes the biosensor as a reaction–diffusion system. For example, when the apparent Michaelis constant $K_{\mathrm{M}}^{\mathrm{app}}$ is much larger than its value for soluble enzyme K_{M}, it means that significant diffusion barrier is present between the sample and the reaction layer [1–8]. These statements on the meaning of $K_{\mathrm{M}}^{\mathrm{app}}$ are considered to be clear for the case of the planar electrodes. Otherwise, there is a class of non-planar biosensors, which are based on bulk modification of entire electrode material, e.g. enzyme modified porous carbon electrodes [9]. What is a meaning of apparent Michaelis constant in this specific case? Some preliminary data on this matter have been presented in our recent papers on the mathematical modeling of porous electrodes [10–12]. It was shown, for example, that apparent Michaelis constant in the certain region of parameters linearly depends on the maximal rate of the enzymatic reaction, i.e. it is not real constant.

The aim of present work was computer simulation of steady state currents at enzyme modified porous electrode using plate-gap electrode model [10], calculation of apparent Michaelis constant for modeled electrode and evaluation of its possible physical meaning. We were also intended to discuss possible applications of proposed model while investigating real biosensors.

2. Materials and methods

The screen-printed carbon electrodes (CE) were designed using carbon black (CB) samples mixed with the pasting liquid as described previously [13]. The pasting liquid consists of 10% polyvinyl dichloride in acetone. A content of amount and assortment of functional groups but not a roughness of CB samples (C1–C12) varies in a wide range [13].

The membrane-bound m-PQQ-ADH purified from *Gluconobacter* sp. 33 (the specific activity was 38.4 U/mg) or soluble type s-PQQ-ADH from *Pseudomonas putida* HK5 (the specific activity was 2 U/mg) were immobilized on the electrode (working area 0.125 cm^2) by adsorption of 3 μl of enzyme solution for 30 min.

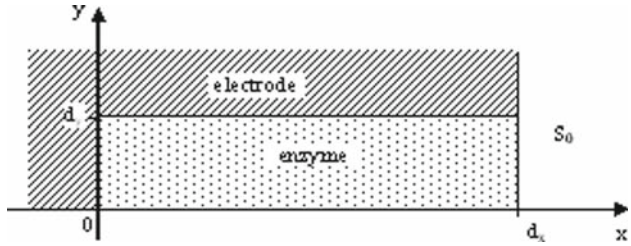

Figure 1. The profile of the biosensor electrode.

All electrochemical measurements were performed using a conventional three-electrode system containing a screen-printed carbon electrode as a working electrode, a platinum wire as a counter electrode and an Ag/AgCl in saturated KCl as a reference electrode (all potential values presented in the text are vs. this reference electrode). About 0.05 M acetate buffer (pH 6.0) containing 1 mM of Ca^{2+} was used as a default buffer. All inorganic reagents were of analytical reagent grade and organic reagents were suitable for electron microscopy. Bidistilled and ultra-filtered water was obtained using a "Purator Bi" (Glas Keramic, Berlin, Germany).

Steady state currents of the biosensors were recorded using a polarographic analyzer PGZ 402 (Radiometer Analytical, France).

Dependences of experimentally measured steady state currents (I) on concentrations of substrates of enzymatic reactions (S) were fitted by hypherbolic function (1). Each experiment was performed in triplicate and mean values were used for curve fitting.

3. Model of enzyme doped porous electrode

It was shown recently, that reaction diffusion conditions in pores of bulk electrode resemble particular conditions in thin gap between parallel conducting plates [10–12]. Here we present the key characteristics of our plate-gap electrode sketched in figure 1.

Firstly, it was assumed, that during an enzyme-catalyzed reaction the substrate S is converted to product P. In the simplest case, the mathematical expression of enzyme kinetics is given by Michaelis–Menten equation:

$$\nu = \frac{\mathrm{d}P}{\mathrm{d}t} = -\frac{\mathrm{d}S}{\mathrm{d}t} = \frac{V_{\max} S}{K_M + S} \tag{3}$$

where $\nu = \nu(S)$ is the rate enzymatic reaction, $V_{\max}$ is the maximal enzymatic rate attainable with that amount of enzyme, when the enzyme is fully saturated with substrate, K_{M} is the Michaelis constant, and t is time.

The dynamics of the considered biosensor system can be described by the reaction–diffusion system

$$\frac{\partial S}{\partial t} = D_f\left(\frac{\partial^2 S}{\partial x^2} + \frac{\partial^2 S}{\partial y^2}\right) - \frac{V_{\max} S}{K_M + S}, \quad 0 < x < d_x, \quad 0 < y < d_y, \quad 0 < t \leqslant T, \tag{4}$$

$$\frac{\partial P}{\partial t} = D_f\left(\frac{\partial^2 P}{\partial x^2} + \frac{\partial^2 P}{\partial y^2}\right) + \frac{V_{\max} S}{K_M + S}, \quad 0 < x < d_x, \quad 0 < y < d_y, \quad 0 < t \leqslant T, \tag{5}$$

where $S = S(x, y, t)$ is the substrate concentration, $P = P(x, y, t)$ is concentration of the reaction product, D_f is the diffusion coefficient of substrate (product) in the gap, d_x is depth of gap; $2d_y$ is gap width; T is full time of biosensor operation to be analyzed.

The operation of biosensor starts when some substrate appears over the surface of enzyme layer. This is used in the initial conditions ($t = 0$)

$$S(d_x, y, 0) = S_0, \quad 0 \leqslant y \leqslant d_y, \tag{6}$$

$$S(x, y, 0) = 0, \quad 0 \leqslant x < d_x, \quad 0 \leqslant y \leqslant d_y, \tag{7}$$

$$P(x, y, 0) = 0, \quad 0 \leqslant x \leqslant d_x, 0 \leqslant y \leqslant d_y, \tag{8}$$

where S_0 is the concentration of substrate in the bulk solution.

The boundary conditions ($0 < t \leqslant T$) are

$$S(d_x, y, t) = S_0, \quad 0 \leqslant y \leqslant d_y, \tag{9}$$

$$P(d_x, y, t) = 0, \quad 0 \leqslant y \leqslant d_y, \tag{10}$$

$$P(0, y, t) = 0, \quad 0 \leqslant y \leqslant d_y, \tag{11}$$

$$P(x, d_y, t) = 0, \quad 0 \leqslant x \leqslant d_x, \tag{12}$$

$$\left.\frac{\partial S(t, x, y)}{\partial x}\right|_{x=0} = 0, \quad 0 \leqslant y \leqslant d_y, \tag{13}$$

$$\left.\frac{\partial S(t, x, y)}{\partial y}\right|_{y=d_y} = 0, \quad 0 \leqslant x \leqslant d_x, \tag{14}$$

$$\left.\frac{\partial S(t,x,y)}{\partial y}\right|_{y=0} = 0, \quad 0 \leqslant x \leqslant d_x, \tag{15}$$

$$\left.\frac{\partial P(t,x,y)}{\partial y}\right|_{y=0} = 0, \quad 0 \leqslant x \leqslant d_x. \tag{16}$$

The current is measured as a response of biosensor in a physical experiment. The current depends upon the flux of reaction product at the electrode surface ($x = 0, 0 \leqslant y \leqslant d_y$ and $y = d_y, 0 \leqslant x \leqslant d_x$). Consequently, a density $I(t)$ of the current at time t is proportional to the concentration gradient of the product at the surface of the electrode as described by Faraday's law:

$$I(t) = n_{\mathrm{e}} F D_f \left(\int_0^{d_y} \left.\frac{\partial P}{\partial x}\right|_{x=0} \mathrm{d}y + \int_0^{d_x} \left.\frac{\partial P}{\partial y}\right|_{y=d_y} \mathrm{d}x \right) \tag{17}$$

where n_e is number of electrons involved is a charge transfer at the electrode surface, and F is Faraday constant, $F \approx 9.65 \times 10^5\,\mathrm{C/mol}$. The steady state current I of the plate-gap electrode is defined as:

$$I = \lim_{t \to \infty} I(t) \tag{18}$$

Digital simulation carried out in [10,11] led to approximate relationships between given and apparent parameters of the plate-gap electrode

$$K_M^{\mathrm{app}}/K_M \approx 1 + C V_{\max} d_x^2 / K_{\mathrm{M}} D_{\mathrm{f}}, \tag{19}$$

where C is constant. Approximate equation (19) was valid for the sets of used parameters, which resulted in $K_M^{\mathrm{app}}/K_{\mathrm{M}} < 10$. Significant deviation from validity of (19) was observed when diffusion time representing factor (d_x^2/D_f) was large [11]. One can guess that similar deviations from approximate equation (19) may be achieved also by rising reaction rate $V_{\max}$. In further digital simulations, we are intended to check the validity of such presumption.

4. Digital simulation

The following values of the parameters were constant in the numerical simulation of all the experiments

$$D_f = 10^{-6}\mathrm{cm}^2/\mathrm{s}, \quad d_x = 5 \cdot 10^{-3}\mathrm{cm}, \quad 2d_y = 10^{-3}\mathrm{cm}, \quad n_e = 2. \tag{20}$$

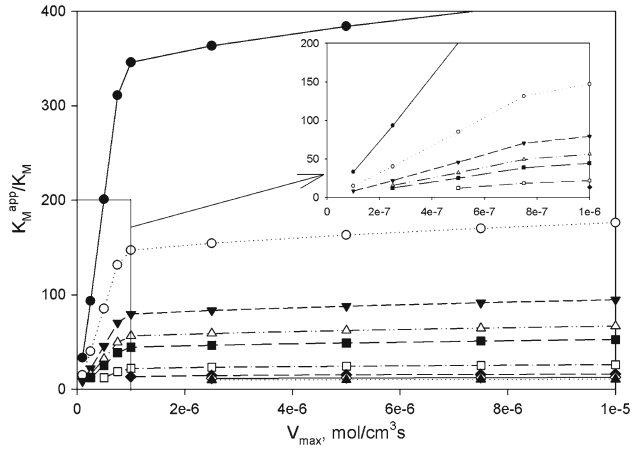

Figure 2. Dependences of dimensionless parameter K_M^{app}/K_M on the maximal reaction rate for different values of $K_M.K_M$ ranged from 10^{-8} (top curve) to 10^{-6} (bottom curve) mol/cm^3.

Numerical solution of the model was evaluated for different values of the parameters $V_{max}(10^{-7}, 2.5 \cdot 10^{-7}, 5 \cdot 10^{-7}, 7.5 \cdot 10^{-7}, 10^{-6}, 2.5 \cdot 10^{-6}, 5 \cdot 10^{-6}, 7.5 \cdot 10^{-6}, 10^{-6}$ mol/cm^3 s), $K_M(10^{-8}, 2.5 \cdot 10^{-8}, 5 \cdot 10^{-8}, 7.5 \cdot 10^{-8}, 10^{-7}, 2.5 \cdot 10^{-7}, 5 \cdot 10^{-7}, 7.5 \cdot 10^{-7}, 10^{-6}$ mol/cm^3). Steady state currents were calculated for modeled biosensors responding to various substrate concentrations $S_0(10^{-7}, 2.5 \cdot 10^{-7}, 5 \cdot 10^{-7}, 10^{-6}, 2.5 \cdot 10^{-6}, 5 \cdot 10^{-6}, 10^{-5}$mol/cm^3). Values of all selected parameters are of the orders that are typical for real amperometric biosensors. Each calibration curve was approximated by hyperbolas (1) and apparent K_M^{app} of modeled amperometric biosensor were calculated.

Dependences of apparent Michaelis constant on the maximal reaction rate (figure 2) contain two distinct regions. The linear one obeys previously derived equation (19). The region of saturation is achieved at sufficiently high values of V_{max}. In this case, apparent Michaelis constant is just slightly dependent on the maximal reaction rate.

Correlations between apparent parameters of plate-gap electrode are shown on figure 3. As can be seen on this figure K_M^{app} is linearly dependent on I_{max}^{app} when $I_{max}^{app} < 100$ nA. For $I_{max}^{app} > 100$ nA saturation process resulting in almost constant values of apparent Michaelis constant is observed. The dependences of K_M^{app} vs. V_{max} or K_M^{app} vs. I_{max}^{app} can be characterized by the specific spatial distribution of substrate, product and catalytic current in the gap. When biosensor operates in the linear regime, reaction spans the whole gap (figure 4). Contrarily, in saturation regime current is concentrated on the top of the gap (figure 5). Such electrode looks as the heterogeneous system, where reaction and diffusion zones are clearly separated.

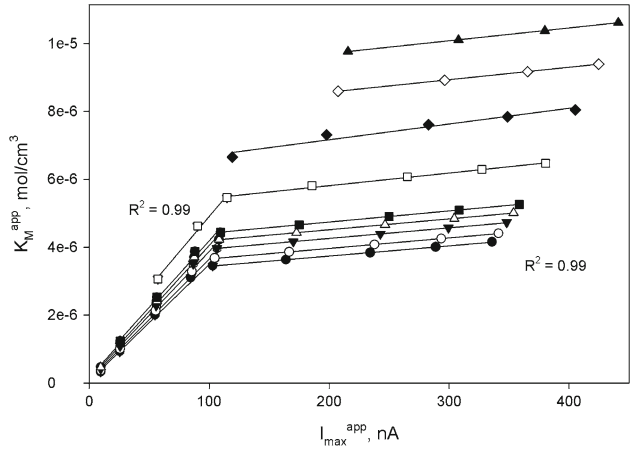

Figure 3. Correlation between apparent parameters of biosensor for different values of K_M. K_M ranged from 10^{-8} (bottom curve) to 10^{-6} (top curve) mol/cm^3.

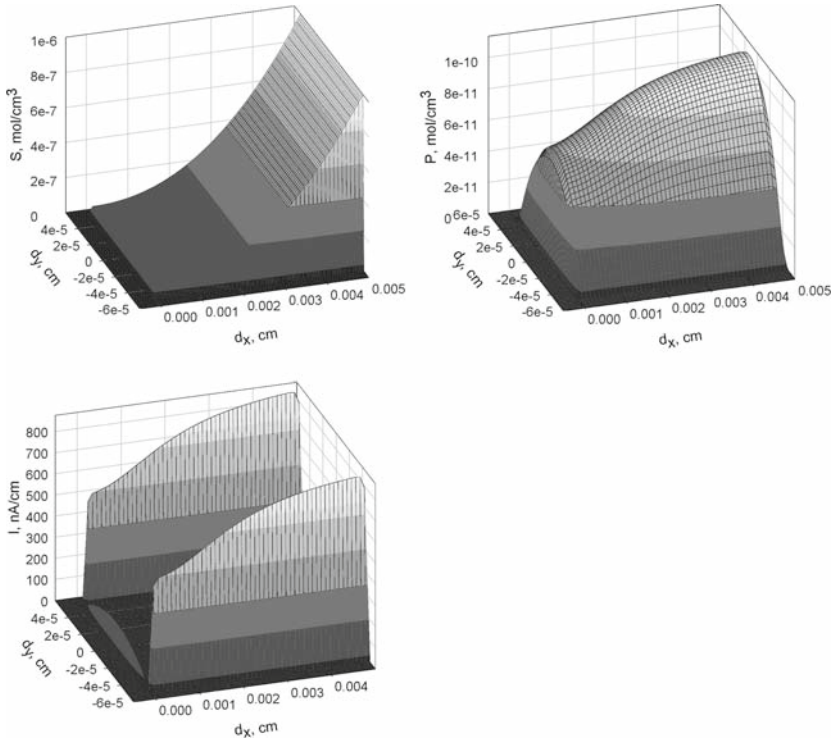

Figure 4. Characteristic distributions of substrate (S), product (P) and current density (I) in plate-gap electrode, which operates in linear regime ($K_{\mathrm{M}} = 5 \times 10^{-8}$ mol/cm^3; $V_{\max} = 10^{-8}$ mol/cm^{3}s).

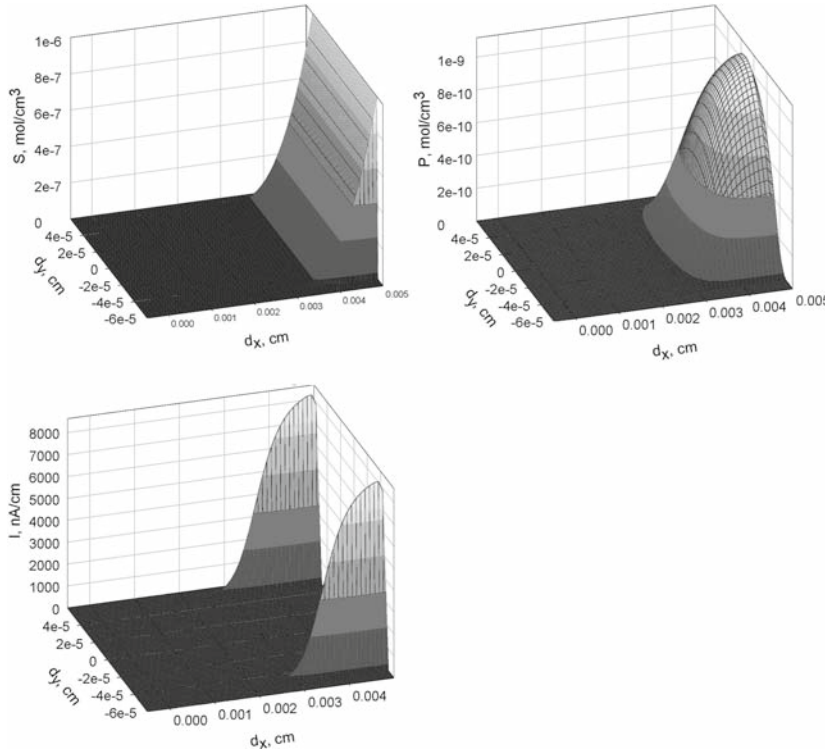

Figure 5. Characteristic distributions of substrate (S), product (P) and current density (I) in plate-gap electrode, which operates in saturation regime ($K_{\mathrm{M}} = 5 \times 10^{-8}\,\mathrm{mol/cm^3}$; $V_{\max} = 10^{-7}\,\mathrm{mol/cm^3 s}$).

5. Apparent parameters of carbon paste electrodes

Experimentally obtained correlations between apparent parameters of enzyme modified carbon paste electrodes are shown on figures. 6 and 7. The correlations between $K_{\mathrm{M}}^{\mathrm{app}}$ and $I_{\max}^{\mathrm{app}}$ seem to be close to linear. The observed dependences correspond to linear regions of theoretically calculated dependences shown in figure 3. The saturation regime was not observed due to, probably, too low activity of immobilized enzymes.

6. Discussion

Mathematical modeling and digital simulation of biosensor make sense if these theoretical approaches are capable to explain experimental results or indicates the recipes how to improve operation of these devices. Let us summarize the results of our calculations emphasizing their fitness to real biosensors.

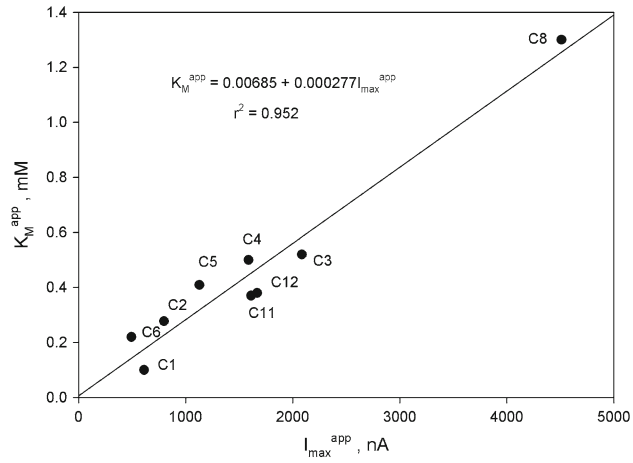

Figure 6. Correlation between apparent parameters of ethanol biosensor based on m-PQQ-ADH.

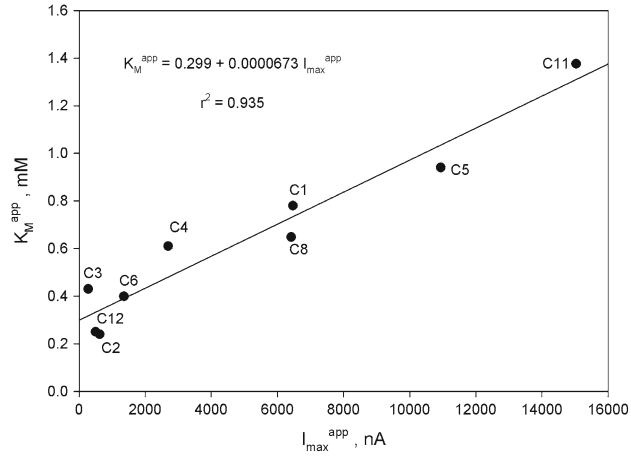

Figure 7. Correlation between apparent parameters of ethanol biosensor based on s-PQQ-ADH.

Firstly, in the frame of our model, the approximate dependences of apparent Michaelis constant on apparent maximal current contain two distinct regions:

$$K_{\mathrm{M}}^{\mathrm{app}}/K_{\mathrm{M}} \approx 1 + a I_{\mathrm{max}}^{\mathrm{app}}, \quad \text{when } I_{\mathrm{max}}^{\mathrm{app}} < I_{\mathrm{max}}^{\mathrm{app}}(\mathrm{sat}) \tag{21}$$

$$K_{\mathrm{M}}^{\mathrm{app}}/K_{\mathrm{M}} \approx 1 + a I_{\mathrm{max}}^{\mathrm{app}}(\mathrm{sat}), \quad \text{when} I_{\mathrm{max}}^{\mathrm{app}} > I_{\mathrm{max}}^{\mathrm{app}}(\mathrm{sat}) \tag{22}$$

where a is constant. In above equations $I_{\mathrm{max}}^{\mathrm{app}}$ (sat) is$I_{\mathrm{max}}^{\mathrm{app}}$ at saturation point. It follows from (21) that if one observes experimentally gradual decrease of $K_{\mathrm{M}}^{\mathrm{app}}$ when activity of enzyme decreases, this means that biosensor operates in the

linear regime. Such behaviors of carbon paste based working electrodes were reported in the several papers [10–15] and in the current work. In the most of other works on porous electrodes the potential dependences $K_{\mathrm{M}}^{\mathrm{app}}$ on $I_{\max}^{\mathrm{app}}$ were passed over in silence. Our calculations and experimental measurements show that apparent parameters must be dependent on each other, when biosensor operates below saturation regime. Linear dependence (21) indicates that apparent Michaelis constant as well as apparent maximal current is a measure of both key features of particular biosensor: linearity of calibration curve and sensitivity. On the other hand, surprisingly, apparent Michaelis constant is practically independent on K_M of soluble enzyme. For example, 100-fold decrease of K_M results just in two-fold decrease of $K_{\mathrm{M}}^{\mathrm{app}}$ (figure 3). Therefore, apparent Michaelis constant can be regarded as biosensor's topology related technological parameter, which is practically not related to the specificity of enzyme kinetics.

The plate-gap electrode in saturation regime behaves like planar electrode, which contains a diffusion layer [1]. In both cases, relatively high values of apparent Michaelis constants theoretically can be achieved. One can notice that plate-gap electrode operating in saturation regime mimics the structure of planar electrode, which contains diffusion membrane. It looks like "inverted" planar electrode where 'apparent' diffusion layer is below the reaction layer (figure 5). In this case, reaction proceeds on the top of the gap. Consequent formation of apparent diffusion layer results in the highest values of apparent Michaelis constant like in the case of enzymatic planar electrodes covered with diffusion membrane. Seemingly, for the both model systems the high values of apparent Michaelis constant indicate stratification of the reaction–diffusion system into separate reaction and diffusion 'zones'. Results of our work show that the specific topology of the reaction–diffusion system cannot be recognized from calibration curve analysis. Planar electrode covered by diffusion membrane and plate-gap electrodes behaves and 'looks' similarly. Therefore, theoretically, there should be at least two alternative technological approaches to achieve higher apparent Michaelis constant and better linearity of the biosensor. The first, classical way is to form diffusion layer on the top of flat reaction layer [1–8]. The second is to form apparent diffusion layer below reaction layer using properties of porous conducting materials. In the frame of our model, the effect of porosity of conducting materials on apparent Michaelis constant should be nearly equivalent to the effect of diffusion membrane for planar electrode.

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References

[1] A.P.F. Turner, I. Karube and G.S. Wilson, *Biosensors: Fundamentals and Applications* (Oxford University Press, Oxford, 1987).
[2] G.G. Guilboult and G. Nagy, Anal. Chem. 45 (1973) 417.
[3] L.D. Mell and J.T. Maloy, Anal. Chem. 47 (1975) 299.
[4] L.D. Mell and J.T. Maloy, Anal. Chem. 48 (1976) 1597.
[5] J. Kulys, V.V. Sorochinski and R.A. Vidziunaite, Biosensors 2 (1986) 135.
[6] T. Schulmeister, J. Rose and F. Scheller, Biosens. Bioelectron. 12 (1997) 1021.
[7] R. Baronas, F. Ivanauskas, J. Kulys and M. Sapagovas, J. Math. Chem. 34 (2003) 227.
[8] D.R. Thevenot, K. Toth, R.A. Durst and G.S. Wilson, Pure Appl. Chem. 71 (1999) 2333.
[9] L. Gorton, Electroanalysis 7 (1995) 23.
[10] F. Ivanauskas, I. Kaunietis, V. Laurinavicius, J. Razumiene and R. Simkus, J. Math. Chem. 38 (2005) 355.
[11] I. Kaunietis, R. Simkus, V.Laurinavicius and F. Ivanauskas, Nonlinear Anal. Modelling Control 10 (2005) 211.
[12] R. Baronas, F. Ivanauskas and I. Kaunietis and V. Laurinavicius, Sensors 6 (2006) 727.
[13] J. Razumiene, J. Barkauskas, V. Kubilius, R. Meskys and V. Laurinavicius, Talanta 67 (2005) 783.
[14] J. Razumiene, A. Vilkanauskyte, V. Gureviciene, J. Barauskas, R. Meskys and V. Laurinavicius, Electrochim. Acta 51 (2006) 5150.
[15] R. Garjonyte and A. Malinauskas, Sens. Actuator B-Chem. 96 (2003) 509.