VILNIUS UNIVERSITY

Mantas Puida

COMPUTER MODELING OF STRUCTURAL INNOVATIONS IN BIOSENSORS

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VILNIAUS UNIVERSITETAS

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Contents

1 Introduction

Computer modeling is a very important method of scientific research. This method plays an important role in the fields where several different disciplines of science meet together. Multidisciplinary fields are the most suitable place to reveal the best features of computer modeling. These best features include saving human and physical resources, quantum improvement of system knowledge, also discovery of new knowledge that sometimes cannot be acquired by direct physical experiments. Biosensors are such multidisciplinary field where computer modeling can speed up the research. Biosensors are small analytical devices widely used for environment analysis and control of complex biotechnological processes or even bioterrorism prevention. Continuous extension of the field of their application and improvement of existing biosensors allow to improve the quantity and quality of industrial products, health care and security. As mentioned above, this is the field where multiple disciplines are meeting together: physics, chemistry, mathematics and informatics. Processes happening inside the biosensor, like electrical current and diffusion, belong to the field of physics. Other processes, like enzyme binding to the substrate and turning it into product, belong to field of biochemistry. Mathematics is used to describe these processes in a language of equations that describe the quantities and relationships among the reacting components. Only simple cases of these equations could be solved analytically, so these more complex cases need to be solved using numeric methods on computers, in other words, computer modeling is performed. Close collaboration of these sciences is a key to successful improvement of biosensors.

1.1 Field of study

Biosensors are one of the rapidly changing fields of research and application. As already mentioned above, biosensors are analytical devices

made from bioactive substance, which reacts with analyte and generates signal, signal detection or conversion subsystem, which transforms signal into a more convenient form [Schell92, Blum91]. Enzymes, antibodies or even whole cells could be used as the bioactive element. Electrodes, photo elements, etc. serve as signal change subsystems. Enzymatic biosensors are the most popular ones.

Today biosensors are used in various areas of life: healthcare, environment control, bioterrorism prevention, pathogen and toxin detection, food, paper and detergent industries. Usually they are used when there is no access to laboratory equipment or long analysis duration is not feasible. Biosensors make good candidates for this task, because they are small, mobile, sensitive and fast [Schmi98, Born99, Houde04, Blum91]. Continuous improvement of biosensors remains an important problem, because it allows to expand the field of application of biosensors. Structural biosensor innovations, which allow producing implantable biosensors, are good examples of the importance of the research of new [Tran93, Yang06, Yu06].

Another example of structural biosensor innovation is a biosensor for assessing activity of triacylglycerol hydrolases (EC 3.1.1.3) that cleave triacylglycerols at the oil/water interface, have extensive applications in the food, paper, pharmaceutical, cosmetic, detergent, leather, and textile industries [Schmi98, Houde04]. Usually enzyme activity is assessed by titration methodology, which requires laboratory equipment and thus sometimes it is not feasible. A novel method for assessing lipase activity was described in the paper [Ignat05]. The work in question discusses how a lipid-like synthetic compound O-palmitoyl-2,3-dicyanohydroquinone (PDCHQ), that contains both the ester and the electroactive hydroquinone-based groups, was used as a lipase substrate. The PDCHQ molecules were solubilized in the Triton X-100 micelles, while the product of enzymatic hydrolysis, 2,3-dicyanohydroquinone, was readily oxidized on the electrode in a diffusion-controlled process. The magnitude of the electrode current is determined solely by the concentration

and diffusion coefficient of the electroactive species, thus proportional to the activity of the enzyme [Ignat05, Bard01].

Another novel electrochemical technique for the assay of lipase activity has been described by [Valin05]. The method utilizes a solid supported lipase substrate, which is formed by dripping and drying a small amount of an ethanol solution of 9-(5'-ferrocenylpentanoyloxy)nonyl disulfide (FPONDS; $[Fc\text{-}(CH_2)_4COO(CH_2)_9S\text{-}]_2$, where Fc is the ferrocene) on the gold electrode surface modified by a hexanethiol self-assembled monolayer. The redox-active ferrocene group of FPONDS generates the amperometric signal, the intensity of which is proportional to the number of FPONDS molecules at the interface. Electrochemical signal decay rate is proportional to the enzyme activity.

Biosensors described above are distinguishable from other biosensors by use of substrate as bioactive component instead of enzyme. This is a structural innovation. Controllable permeability membrane could be seen as another structural biosensor innovation. Theoretical premises for such biosensor do exist, i.e. there are substances that change their permeability depending on received charge or medium pH [Shimi88]. Such theoretical premises still need to be verified. One of ways to perform such analysis is the application of mathematical and computer modeling.

Computer modeling is one of most important tools used to continuously create new and improve the already existing biosensors. The main goal of computer modeling is to identify which factors (e.g. chemical reaction rate, diffusion rate, activity of the bioactive element) are the most important for the biosensor's response to the concentration of analyte. A mathematical model describes physical and chemical processes that happen inside of the biosensor. Computer simulation performed according to this model allows to observe and interact with these processes at a desired scale. Usually several processes are happening inside a biosensor simultaneously, like the interaction of the enzyme and the substrate, dissipation of the formed complex, the oxidation/reduction of the reaction product on the electrode, diffusion of all acting substances. Relative importance of each of these processes cannot be evaluated with single

experiment [Coop04]. Series of such experiments with variable parameters in comparison to the kinetic model allows to obtain more extensive knowledge of the system in question. Mathematical model and computer simulation are the instruments that allow to plan the future experiments and improve biosensor parameters so that they are better fitted specific application. If there were no mathematical model, the researchers should perform massive amount of physical experiments and go through trials and errors to achieve the same amount of knowledge about how the system works [Coop04]. Computer modeling saves time and resources required for physical experiments and allows to expand the knowledge on how the system works, which is a key to successful improvement of biosensors.

1.2 Specific aims

- Select and apply mathematical and computer models for a lipase activity assessing biosensor with the substrate solubilized in micelles. Verify applicability of the selected model. Analyze what would be the biosensor response time if the sensor works under the selected model.
- Select and apply mathematical and computer models for lipase activity assessing biosensor with the electrode supported substrate. Verify applicability of the selected model. Analyze what would be specific biosensor features and parameters if it works under the selected model.
- Perform computer modeling and analyze the effect of replacing the static membrane with a controllable permeability membrane on biosensor response.

1.3 Scientific novelty

The computer simulation was applied to evaluate the enzymatic reaction rate constant for lipase (Thermomyces lanuginosus) activity assessing biosensor with substrate (O-palmitoyl-2,3-dicyanohydroquinone) solubilized in micelles basing on computer simulation results. It turned out that the constant substrate concentration cannot be assumed, and the kinetic equation system described in [Verger72] should be extended by adding the substrate kinetic equation.

The proposed original kinetic model extension with non-linear second order term for lipase activity assessment biosensor with the electrode supported substrate yielded better results than the classical one. By analyzing the results of the physical experiment it was discovered that the substrate concentration on the electrode decay is expressed in two types of dependencies on time, the exponential one, and the one proportional to t^{-1}.

A mathematical model for original biosensor with controllable permeability membrane was proposed. The proposed original model takes into account medium pH and temperature. It was discovered that using a membrane the permeability of which nonlinearly changes depending on the medium pH, the biosensor could easily be switched from kinetic to diffusion mode, and the linear response range could be extended by several magnitudes. It was shown that a membrane the permeability of which is controllable in time could be superior to the static membrane when the biosensor operates in the electrochemical stripping mode, and the reaction product inhibition to the enzymatic reaction is low.

1.4 Practical value

Presented findings on the specific features of the two novel lipase activity assessing biosensors make ground for the evaluation of the feasibility of the production of such sensors . A detailed analysis of the innovative biosensor

with controllable permeability membrane shows that production of such biosensors might be feasible in some specific cases.

1.5 Findings presented for defense

1. The constant substrate concentration on the micelle surface cannot be assumed for the lipase activity assessing biosensor with the substrate solubilized in micelles. The kinetic model originally described by [Verger72] and extended with the substrate kinetic equation allows for a good fit of physical experiment and computer simulation results when it is assumed that the biosensor is a closed system.
2. The proposed original kinetic model extension with the second order nonlinear substrate wash off term for the lipase activity assessing biosensor with the electrode supported substrate yields better fitting with physical experiment data than the classic reaction model.
3. By replacing the biosensor's static membrane with a membrane the permeability of which nonlinearly depends on medium pH, it is possible to produce a biosensor that would be easily reconfigurable (by switching between kinetic and diffusion modes) between very sensitive and wide linear response range modes.
4. The biosensor with a membrane that controllably changes its permeability in time would be superior to a biosensor with a static membrane, assuming that the biosensor operates in an electrochemical stripping mode and the enzymatic reaction inhibition by the reaction product is low.

2 Computer modeling of lipase activity detection biosensor with substrate solubilized in micelles

2.1 Introduction

Recently, the amperometric detection method of *Thermomyces lanuginosus* lipase activity has been published [Ignat05]. Lipases, triacylglycerol hydrolases (EC 3.1.1.3) that cleave triacylglycerols at the oil/water interface, have extensive applications in the food, paper, pharmaceutical, cosmetic, detergent, leather, and textile industries [Schmi98, Houde04]. Widespread practical use of these enzymes requires fast and reliable analytical routines to assess their activity. The electrochemical technique, described in [Ignat05], presents the method of this kind.

In the work under discussion, a lipid-like synthetic compound O-palmitoyl-2,3-dicyanohydroquinone (PDCHQ), that contains both the ester and the electroactive hydroquinone-based groups, was used as a lipase substrate. The PDCHQ molecules were solubilized in the Triton X-100 micelles, while the product of enzymatic hydrolysis, 2,3-dicyanohydroquinone, was readily oxidized on the electrode in a diffusion-controlled process. Under the diffusion control, the magnitude of the electrode current is determined solely by the concentration and diffusion coefficient of the electroactive species (in the case of work [Ignat05], 2,3-dicyanohydroquinone) and the effective thickness of the diffusion layer [Bard01].

The authors of the paper [Ignat05] have performed experiments under the steady-state conditions. The aim of the present work is computational modeling of response kinetics of this bioelectroanalytical system.

2.2 Physical model

In a simplified one-dimension model (Fig. 2.), the working space of bioelectroanalytical system described in [Ignat05] could be divided into two parts: the first one - wide area, where enzymatic reaction and molecular/particle (convective-)diffusion occur, the second one - narrow area

of a diffusion layer, where the diffusion of hydrolysis product occurs. The latter model assumes that area 2 experimentally could be made inaccessible (e. g., by covering the electrode surface with dialysis membrane) for other components of the system.

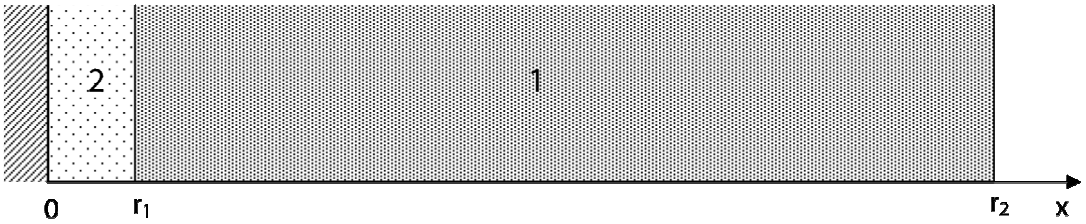


Fig. 2.1 Scheme of the model used in the present study: 1 - area, where reaction and diffusion and/or convection occur, 2 - area, where reaction product diffusion occurs, x=0 - the electrode surface, x=r_1 - outer surface of area 2.

The processes in area 1 could be written in the following schematic form which is most commonly used for the description of lipase interfacial activation [Verger72]:

$$E \underset{k_d}{\overset{k_p}{\rightleftarrows}} E^*, \tag{2.1}$$

$$E^* + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E^*S \xrightarrow{k_{cat}} E^* + P, \tag{2.2}$$

where E is the enzyme in solution, E* is the enzyme penetrated in the surface of micelle, S is the substrate on the micelle surface, E*S is the enzyme-substrate complex, and P represents the reaction product. According to the model, only P diffusion takes place in area 2, generating amperometric response of the system. The electrical signal is proportional to the derivative of reaction product concentration ($\left.\frac{\partial P}{\partial x}\right|_{x=0}$). The change of this parameter with time is the object of computational simulations.

2.3 Mathematical model

The system under discussion can be described by two different mathematical models:

1. Assuming that area 1 is large enough and substances are distributed evenly, e.g., by convection process. Thus, it may be inferred that the concentrations of all substances are uniform across all area, and reaction equations can be solved in single space point without taking diffusion into account. Also it is assumed that there is no special separation between areas 1 and 2, therefore all substances (except the reaction product P) are uniformly distributed across area 2;

2. It is assumed that substances are distributed non-uniformly in area 1 and diffusion should be taken into account. It is also assumed that there is special separation between areas 1 and 2 (e.g., area 2 represents dialysis membrane of thickness r1 on the electrode surface), so only reaction product diffusion occurs in area 2.

For both models it is true that beyond zone 1 ($x>r_2$), there is large volume uniformly filled with the same substances and where the same reactions occur. All these substances and reaction product flow to zone 1 through boundary $x=r_2$.

First model is described by the following system of non-linear differential equations for single area 1 space point [Verger72]:

$$\frac{dE}{dt} = -k_p \frac{I}{V} E + k_d \frac{I}{V} E^* \tag{2.3}$$

$$\frac{dE^* S}{dt} = k_1 E^* \times S - (k_{cat} + k_{-1}) E^* S \tag{2.4}$$

$$\frac{dE^*}{dt} = k_p E + (k_{cat} + k_{-1}) E^* S - (k_d + k_1 S) E^* \tag{2.5}$$

$$\frac{dS}{dt} = k_{-1} E^* S - k_1 E^* \times S \tag{2.6}$$

where symbols E, E^*, E^*S and S represent concentrations; I is the total interfacial area of micelles; V is the total volume; k_p, k_d, k_1, k_{cat}, k_{-1} are the rate constants shown in Eqs. (2.1) and (2.2); t - time. The following initial conditions (t=0) were applied:

$$E(0) = E_0,$$
$$E^*(0) = 0, E^*S(0) = 0, \tag{2.7}$$
$$S(0) = S_0$$

Additional equation for area 2 (product diffusion plus gain from reactions in this area):

$$\frac{\partial P}{\partial t} = D_p \frac{\partial^2 P}{\partial x^2} + k_{cat} \frac{I}{V} E^* S, \qquad x \in (0, r_1), \tag{2.8}$$

where symbol P represents reaction product concentration; d_p is the diffusion coefficient of P; x - distance, E^*S is calculated from solution of equation system (2.3)-(2.6) . Initial condition (t=0) for the second part of calculations:

$$P(0,x) = 0, \qquad x \in [0, r_1], \tag{2.9}$$

whereas boundary conditions:

$$P(t,0) = 0, \qquad t > 0 \tag{2.10}$$

and

$$\frac{\partial P}{\partial t} = k_{cat} \frac{I}{V} E^* S, \qquad x = r_1, t > 0, \tag{2.11}$$

which is calculated from solution of equation system (2.3)-(2.6).

The second model is described by the system of non-linear partial differential equations [Verger72]:

$$\frac{\partial E}{\partial t} = -k_p \frac{I}{V} E + k_d \frac{I}{V} E^* + D_E \frac{\partial^2 E}{\partial x^2} \tag{2.12}$$

$$\frac{\partial E^* S}{\partial t} = k_1 E^* \times S - (k_{cat} + k_{-1}) E^* S + D_{E^*S} \frac{\partial^2 E^* S}{\partial x^2} \tag{2.13}$$

$$\frac{\partial E^*}{\partial t} = k_p E + (k_{cat} + k_{-1}) E^* S - (k_d + k_1 S) + D_{E^*} \frac{\partial^2 E^*}{\partial x^2} \tag{2.14}$$

$$\frac{\partial S}{\partial t} = k_{-1} E^* S - k_1 E^* \times S + D_S \frac{\partial^2 S}{\partial x^2} \tag{2.15}$$

for area x ∈ (r_1 , r_2). Definitions are the same as for the first model, and d_E, d_{E^*S}, d_{E^*}, d_S are the diffusion coefficients of free enzyme, micellar enzyme-substrate complex, micelle with penetrated enzyme (in fact, d_{E^*S}=d_{E^*}), and substrate, respectively. Reaction product generation and diffusion equation is as follows:

$$\begin{aligned} &\frac{\partial P}{\partial t} = D_P \frac{\partial^2 P}{\partial x^2} + qk_{cat}\frac{I}{V}E^*S, \qquad x \in (0, r_2), \\ &q = \begin{cases} 0 & , x \in (0, r_1]; \\ 1 & , x \in (r_1, r_2). \end{cases} \end{aligned} \tag{2.16}$$

Initial conditions (t=0):

$$\begin{aligned} &E^*(0,x) = 0, E^*S(0,x) = 0, \\ &E(0,x) = E_0, S(0,x) = S_0, x \in [r_1, r_2]; \\ &P(0,x) = 0, x \in [0, r_2]. \end{aligned} \tag{2.17}$$

Boundary conditions:

$$P(t,0) = 0, \qquad t > 0; \tag{2.18}$$

no flow condition for subregions boundary point x = r_1, t > 0:

$$\left.\frac{\partial E}{\partial x}\right|_{x=r_1}(t) = 0, \qquad \left.\frac{\partial S}{\partial x}\right|_{x=r_1}(t) = 0, \qquad \left.\frac{\partial E^*S}{\partial x}\right|_{x=r_1}(t) = 0, \qquad \left.\frac{\partial E^*}{\partial x}\right|_{x=r_1}(t) = 0 \tag{2.19}$$

and boundary condition for point x = r_2 , t > 0:

$$\begin{aligned} &P|_{x=r_2}(t) = \mathrm{Pr}_2(t), \qquad E|_{x=r_2}(t) = Er_2(t), \\ &S|_{x=r_2}(t) = Sr_2(t), \qquad E^*S|_{x=r_2}(t) = E^*Sr_2(t), \\ &E^*|_{x=r_2}(t) = E^*r_2(t). \end{aligned} \tag{2.20}$$

Pr_2 , Er_2 , $\mathrm{E^*r}_2$, $\mathrm{E^*Sr}_2$ are calculated from solution of equation system (2.3)-(2.6).

2.4 Computer simulation setup and results

Dedicated software package was developed to automate computer modeling of the system under study. Matlab was chosen as environment for such software package development. Using this software package the series of computational simulations were performed to investigate how electrode readings would differ if bioelectroanalytical system worked under the first or second model.

The first simulation experiment was designed according to the first model of bioelectroanalytical system. Calculations were divided into two steps: in the first step, calculations were performed according to Eqs. (2.3)-(2.6), and in second step, the diffusion of P was calculated for area 2 according to Eq. (2.8). The following values were used in calculations (all parameters, except kinetic constants, are from [Ignat05]): $D_P = 5.49 \cdot 10^{-5}$ cm^2 s^{-1} , $r_1 = 4 \cdot 10^{-3}$ cm, $I = 7.5 \cdot 10^5$ cm^2 , $V = 10$ cm^3 , $E_0 = 2.35 \cdot 10^{-8}$ mol cm^{-3} , $k_{cat} = 75$ s^{-1} , $k_1 = 1.12 \cdot 10^9$ cm^2 mol^{-1} s^{-1} , $k_{-1} = 10$ s^{-1} , $k_p = 100$ cm s^{-1} , $k_d = 0.025$ s^{-1} , $S_0 = 6.7 \cdot 10^{-12}$ mol cm^{-2}. First step calculations were performed using Matlab ODE solver for stiff problems. For the second part of calculations the system of differential equations was discretized using the implicit finite difference scheme [Samar01], system of linear equations corresponding to this scheme was solved using matrix solver for tridiagonal matrices. Integration steps in space and time were as follows: $h_x = 5 \cdot 10^{-6} cm$, $h_t = 1 s$; integration in time interval was T=[0..3000]. The results of computational experiment are presented in Fig. 2.2.

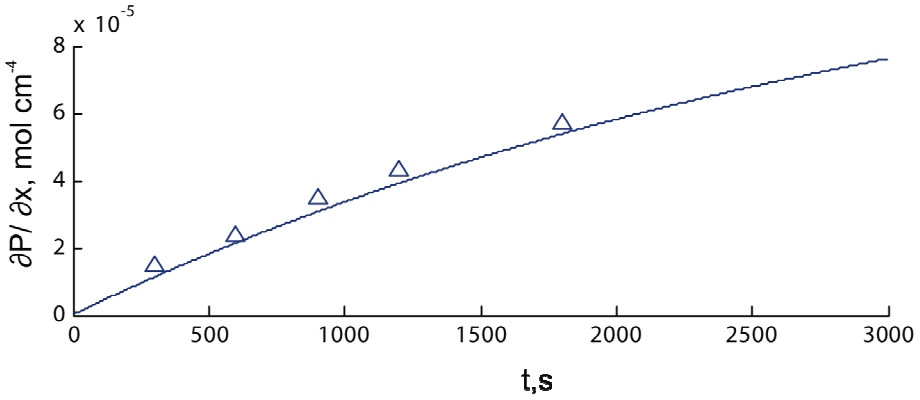


Fig. 2.2 Δ – recalculated experimental data points from [Ignat05] ($\left.\frac{\partial P}{\partial x}\right|_{x=0}$ (in mol cm^{-4}) dependency on time); solid line - computed $\left.\frac{\partial P}{\partial x}\right|_{x=0}$ (in mol cm^{-4}) dependency on time (in *seconds*) according to the first model of bioelectroanalytical system.

Fig. 2.2 also contains the recalculated data points from ref. [Ignat05]. For the rotating disk electrode employed in [Ignat05], $\left.\frac{\partial P}{\partial x}\right|_{x=0} = I/(nFAd_P)$ [Bard01], where I is the experimental current values, n=2 is the number of electrons transferred during the oxidation of P, F is the Faraday constant, and A=0.07 cm^2 is the electrode surface area. As can be seen from Fig. 2.2, the mathematical model (model 1) and a set of kinetic constants used in the computational experiment enabled to attain good agreement between the experimental and modeling results. It is believed that systematically slightly higher values of experimental data points in Fig. 2.2 result from imperfect subtraction of background current in work [Ignat05]. Unfortunately, because of complexity of interfacial lipase action (see Eqs. (2.1) and (2.2)), individual kinetic constants for this enzyme are not reported in the literature. This fact presents difficulties to check the validity of the kinetic constants used in calculations.

The second experiment was designed according to the second model of amperometric system. Constant values were the same as for the first experiment, additionally the second stagnant diffusion layer was defined: $r_2 = 8\times10^{-3} cm$; d_{E^*S}=10^{-7} cm^2 s^{-1}, d_{E^*}=10^{-7} cm^2 s^{-1}, d_E=10^{-6} cm^2 s^{-1},

d_S=10^{-6} cm^2 s^{-1}, when $r_1 \le x \le r_2$ and, for the diffusion coefficients, zero in other cases. Differential equation system was transformed into implicit finite differences scheme and non-linear equation system corresponding to this scheme was solved using simple iterations method [Samar01]. The results are presented in Fig. 2.3 (continuous line). The same experiment was repeated two more times with following boundary location pairs *a*) r_1=8 10^{-4}, r_2=4.8 10^{-3}; *b*) r_1=4 10^{-4}, r_2=4.4 10^{-3} (all values in *cm*).

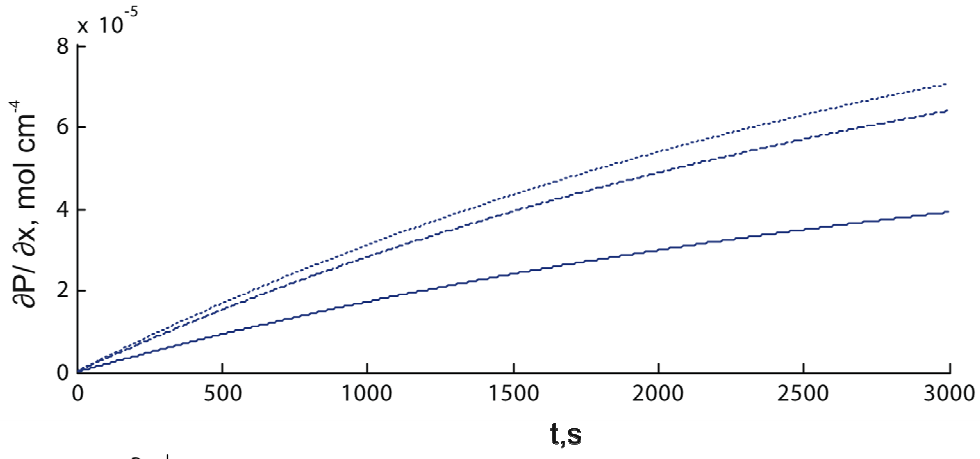


Fig. 2.3 Solid line - $\left.\frac{\partial P}{\partial x}\right|_{x=0}$ (in mol cm^{-4}) dependency on time (in *seconds*) according to the second model of analytical system and assuming r_1=4·10^{-3} *cm* and r_2=8·10^{-3} *cm*; dashed line - r_1=8·10^{-4} *cm* and r_2=4.8·10^{-3} *cm*; and dotted line - r_1=4·10^{-4} *cm* and r_2=4.4·10^{-3} *cm*.

2.5 Conclusions

The results of foregoing computational experiments enable to make the following conclusions:

1. Model described by [Verger72] cannot be applied directly for *Thermomyces lanuginosus* lipase activity assessing biosensor. Constant substrate concentration cannot be assumed thus model should be extended with substrate kinetic equation. Such model extension allows obtaining good fit between experimental and computer simulation results.

2. An enzymatic reaction rate constant for *Thermomyces lanuginosus* lipase with respect to the synthetic substrate, O-palmitoyl-2,3-dicyanohydroquinone, is suggested (in context of set of other individual kinetic constants) in the computational experiments.

3. As expected, modeling of the analytical system with additional stagnant diffusion layer (the diffusion layer in model 1 is replaced by the dialysis membrane of the same thickness, and the second diffusion layer of constant thickness in model 2 is formed, for instance, by electrode rotation) demonstrates a decreased initial rate of system response (i.e., rate of current increase upon enzyme injection in the system). Computational experiments also show that significant decrease of dialysis membrane thickness and increase of electrode rotation rate (leads to the decreased thickness of the second diffusion layer) should improve the performance of the analytical system. Electrochemical experiments along these lines are in progress.

3 Computer modeling of lipase activity detection biosensor with electrode supported substrate

3.1 Introduction

Lipolytic enzymes are one of the most important components of the biochemical processes. At the same time, triacylglycerol acylhydrolases (EC 3.1.1.3) that hydrolyze triacylglycerols at the oil/water interface have wide applications as detergent additives, digestive aids, as well as in the paper and food industries [Schmi98, Born99, Houde04]. Unlike other bond-cleaving enzymes, e.g., proteases, hydrolysis by lipases is carried out in heterogeneous multiphase systems. In many cases, the environment of the enzyme at the substrate/liquid interface plays an important role for the overall enzymatic activity of these proteins [Schmi98, Born99, Houde04, Beiss00]. Thus, the ability to monitor enzymatic activity of lipases under these conditions is of paramount importance.

Recently, a novel electrochemical technique for the assay of lipase activity has been described [Valin05]. The method utilizes a solid supported lipase substrate, which is formed by dripping and drying a small amount of an ethanol solution of 9-(5'-ferrocenylpentanoyloxy)nonyl disulfide (FPONDS; $[Fc\text{-}(CH_2)_4COO(CH_2)_9S\text{-}]_2$, where Fc is the ferrocene) on the gold electrode surface modified by a hexanethiol self-assembled monolayer. The redox-active ferrocene group of FPONDS generates the amperometric signal, the intensity of which is proportional to the number of FPONDS molecules at the interface. Electrochemical and surface-enhanced infrared absorption spectroscopic data, as well as control experiments with an engineered, deactivated mutant enzyme, have demonstrated that the wild-type lipase from *Thermomyces lanuginosus* (TLL) is capable of cleaving the ester bonds of FPONDS molecules via an enzymatic hydrolysis mechanism, which includes the adsorption of the lipase onto the substrate surface. The interfacial enzymatic process liberates ferrocene groups from the electrode surface triggering a decay of the

electrochemical signal. The rate of the electrochemical signal decrease is proportional to the lipase activity.

However, in exclusively experimental work [Valin05], no kinetic model has been proposed to account for the features of amperometric biosensor response, namely, current decay vs. time upon enzyme action. This paper is intended to fill this gap.

3.2 Physical model

This paper analyzes bioelectroanalytical system that is significantly different from recently discussed amperometric system of lipase activity determination, where enzyme acts on the surface of substrate-bearing micelles spread in the solution. Currently modeled system is schematically presented in Fig. 3.1.

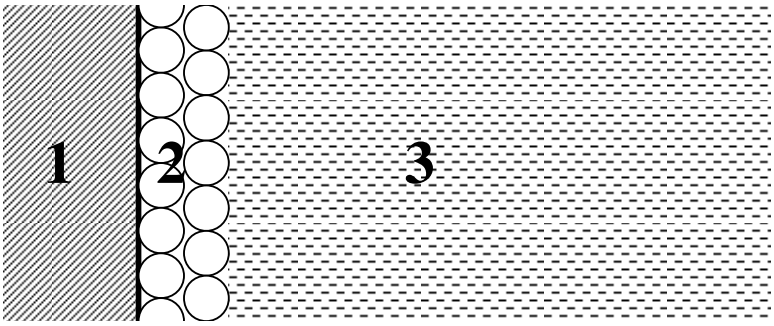


Fig. 3.1. Cross-section scheme of the model used in the present study: 1 – gold electrode, 2 – FPONDS substrate layer(s), 3 – lipase solution.

The processes that occur at the interface of zones 2 and 3 could be described in the following schematic form which is most commonly used for the description of lipase interfacial activation [Verger72]:

$$E \underset{k_D}{\overset{k_p}{\rightleftarrows}} E^* \tag{3.1}$$

$$E^* + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E^*S \xrightarrow{k_2} E^* + P, \tag{3.2}$$

where E is the enzyme in solution, E* is the enzyme attached to the surface of substrate (at the interface of zones 2 and 3 in Fig. 3.1), S is the ferrocene-based substrate FPONDS substrate on the gold electrode surface, E*S is the enzyme-substrate complex, and P represents the reaction product. The change of S concentration as a function time is the object of computational simulations as it is directly proportional to experimentally registered electrode signal (see, for instance, Fig. 1 in [Valin05]).

3.3 Mathematical model

It is assumed that lipase solution is distributed evenly and its diffusion could be not taken into account. It is also assumed that the redox-active reaction product (ferrocene-based) leaves sensor surface quite fast and its diffusion could be estimated as instantaneous. The system under discussion can be described by classical mathematical model of reaction kinetics:

$$\begin{cases} \dfrac{dE^*}{dt} = k_{-1}E^*S + k_2E^*S - k_1E^* \times S + k_pE - k_DE^* \\ \dfrac{dE^*S}{dt} = k_1E^* \times S - k_{-1}E^*S - k_2E^*S \\ \dfrac{dS}{dt} = k_{-1}E^*S - k_1E^* \times S \\ \dfrac{dP}{dt} = k_2E^*S \\ \dfrac{dE}{dt} = \left(\dfrac{I}{V}\right)k_DE^* - \left(\dfrac{I}{V}\right)k_pE \end{cases} \tag{3.3}$$

where symbols in italics *E*, *E**, *E*S*, *P* and *S* represent concentrations; *I* is the interfacial area of electrode; *V* is the total volume of solution; k_p is the rate constant of enzyme adsorption at the electrode surface, k_D is the enzyme desorption rate constant, k_1 is the rate constant of enzyme-substrate complex (E^*S) formation, k_{-1} is the rate constant of E*S dissociation, k_2 is the catalytic rate constant of enzymatic reaction, and *t* is time.

This model allowed good fitting only for a part of experimental data available (data not shown), which had strongly expressed exponential character

of substrate concentration decrease (Fig. 3.2, experiment B; for the characteristics of different experiments, see parameters in the table).

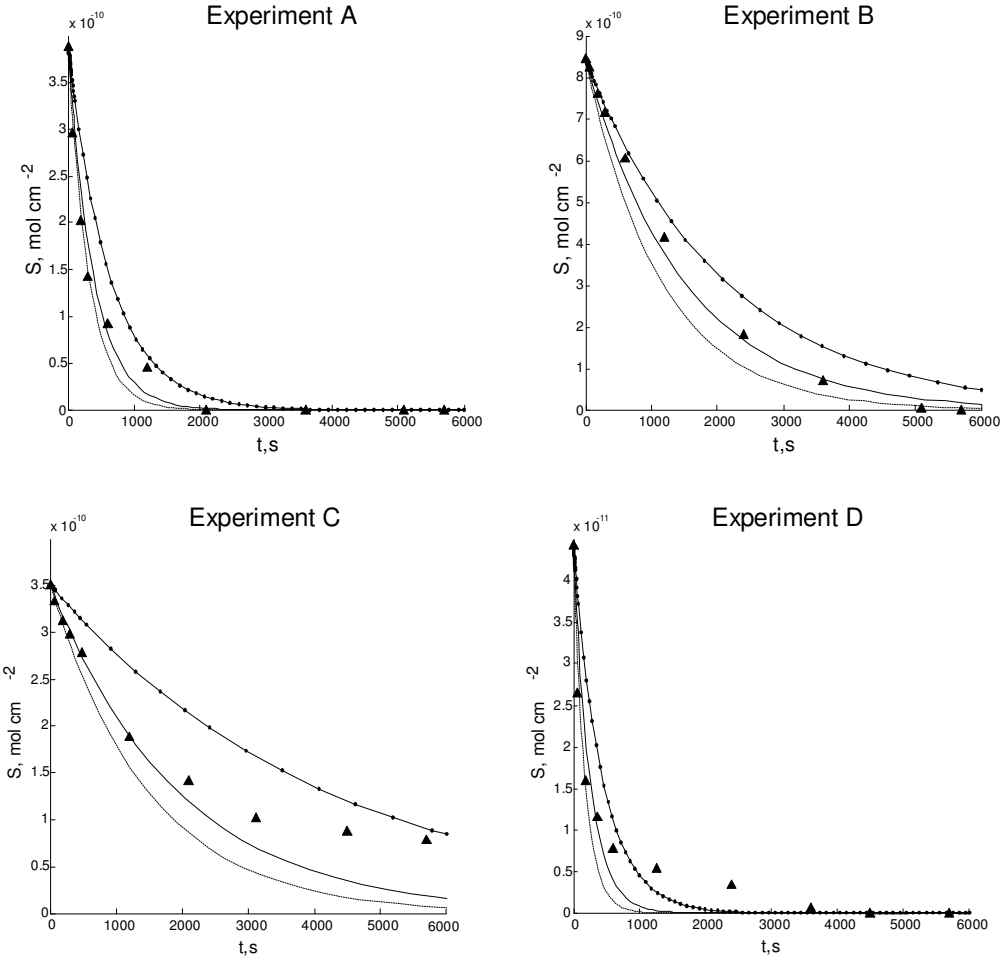


Fig. 3.2. Experiment A and B data analysis: ▲- 1/S dependency on time;▼- ln(S) dependency on time.

However, another part of experimental data exhibited *S* decrease asymptotically proportional to t^{-1} (Fig. 3.2, experiment A). Thus, the model of Eq. (3.3) was modified by adding a non-linear term of substrate wash off from the electrode surface, which allowed much better fitting results. Here, it should be noted that in work [Valin05] the wash off effect of substrate has been observed experimentally in the solutions without added enzyme (see Fig. 1 in paper [Valin05]). Therefore, we have reasonable grounds to believe that this process also occurs in the solutions containing enzyme.

Thus, slightly modified system of non-linear differential equations can be written by Eq. (3.4):

$$\begin{cases} \dfrac{dE^*}{dt} = k_{-1}E^*S + k_2E^*S - k_1E^* \times S + k_pE - k_DE^* \\ \dfrac{dE^*S}{dt} = k_1E^* \times S - k_{-1}E^*S - k_2E^*S \\ \dfrac{dS}{dt} = k_{-1}E^*S - k_1E^* \times S - k_S\left(\dfrac{S}{S_0}\right)^2 \\ \dfrac{dP}{dt} = k_2E^*S \\ \dfrac{dE}{dt} = \left(\dfrac{I}{V}\right)k_DE^* - \left(\dfrac{I}{V}\right)k_pE \end{cases} \tag{3.4}$$

where definitions are the same as for Eq. (3.3), and k_S is the substrate wash off rate constant and S_0 is the initial substrate concentration on the electrode surface.

Non-linear wash off term is quite unusual, but it could be explained in a simplified way as complex outcome of two different linear wash off rates: one for the electrode surface/substrate boundary (stronger bond, lower wash off rate) and second (weaker attraction, much higher wash off rate) for, say, substrate/substrate boundary. It is possible that during the process of modified electrode preparation substrate forms only very few substrate/substrate boundaries (pseudo-multilayer interfacial structure). Thus, initially wash off rate could be seen as linearly (in respect to the substrate concentration) dropping from high value for the substrate/substrate boundary, down to low value for the electrode/ substrate boundary, and the whole process then becomes second order with respect to the substrate concentration. By way of illustration, let's assume that the wash-off rate constant (k) changes linearly with relative substrate concentration: $k=a \cdot S/S_0+b$, where a and b are the constants, so non-linearity could be introduced by substituting the wash-off rate constant in standard linear wash-off model: $dS/dt = -kS$.

3.4 Computer simulation setup and results

Software package described in a chapter above was extended to support extended variety of models, like models assuming additional intermeadiate state in enzymatic reaction or models assuming nonlinear substrate wash off terms. The series of computational simulations were performed using this software package to investigate how electrode readings would differ if this amperometric biosensor worked under presented model and how they would match experimental data (experimental results were obtained as described in [Valin05], converting the integrated electrode peak current of the FPONDS-modified electrode to the surfaces concentration of ferrocene functional groups). The following values were used in calculations: $V = 4$ cm^3, $k_2 = 75$ s^{-1}, $k_{-1} = 10$ s^{-1}, $k_p = 100$ cm s^{-1}, $k_D = 0.025$ s^{-1}, $I_A = 5.07\times10^{-2}$ cm^2, $I_B = 5.19\times10^{-2}$ cm^2, $I_C = 5.23\times10^{-2}$ cm^2, $I_D=5.23\times10^{-2}$ cm^2. The values of four kinetic constants selected as a starting point for modeling were the same as in paper [Puida07]. Besides, the following initial conditions were applied: $E(0) = E_0$, $E^*(0) = E^*S(0) = P(0) = 0$, $S(0) = S_0$. The values of initial E_0 and S_0 concentrations varied from experiment to experiment, k_1 and k_u were subject of change for achieving better fitting (weighted least squares method was used) between experimental and simulation data. Non-linear ordinary equation system (3.4) was solved using Matlab (Matlab Release 14, The MathWorks Inc., Natick, USA) ODE solver for stiff problems. Solution time interval was 0..6000 seconds. The initial concentrations and best-fitted constants are presented in the Table 3.1.

Table 3.1. Initial concentrations and best-fitted constants.

Exp.	$S_0\times10^{10}$, mol cm^{-2}	$E_0\times10^{12}$, mol cm^{-3}	$k_1\times10^{-6}$, mol cm^{-2} s^{-1}	$k_S\times10^{13}$, mol cm^{-2} s^{-1}
A	3.88	58.0	0.41	2.26
B	8.43	5.80	1.20	2.13
C	3.51	0.58	1.17	2.06

D	0.44	8.30	0.75	2.34

Experimental data and simulation results are presented in Fig. 3.3.

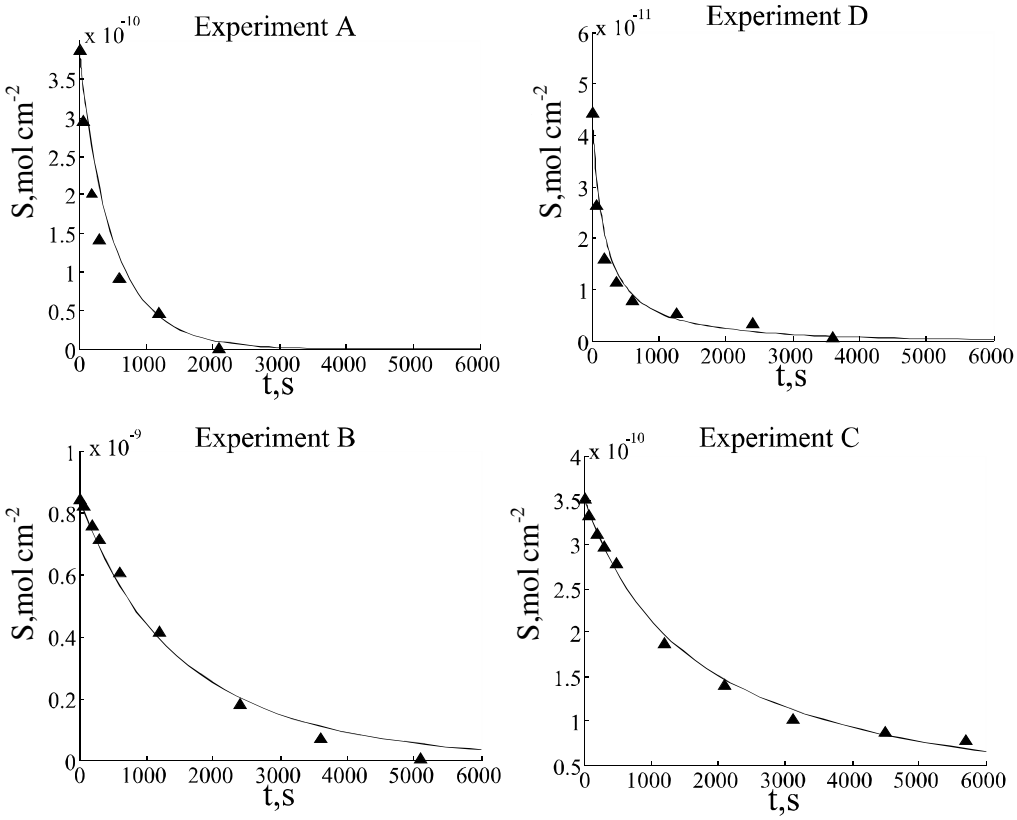


Fig. 3.3. S dependency on time: solid line - simulation results, points – experimental data.

Experimental data were analyzed as logarithmic and t^{-1} graphs. These graphs reveal that experiments A, C and D strongly exhibit inverse dependence on time, whereas the data of experiment B has more exponential character. Such different graph characters could be explained as two term competition in *dS*/*dt* differential equation: first and second order (in respect to the substrate concentration) terms. First order term predominated over the second order term in experiment B, but second order term predominated over the first order term in experiments A, C and D. These observations enabled to improve the model and to get better fitting between simulation and experimental data.

Finally, it is worth noting that the values of kinetic constant k_1 obtained in this work are lower by ca. three orders of magnitude compared to the value

reported in a study [Puida07]. Most likely, the difference is determined by different chemical nature of substrate head-groups in work [Puida07] (dicyanohydroquinone-based group) and the present study (ferrocene-based group), since k_1 reflects molecular event of substrate binding in the enzyme active center.

3.5 Conclusions

The results of the foregoing computational experiments enable to make the following conclusions:

1. The proposed reaction kinetic model of response of the FPONDS-based electrode, used for the electrochemical determination of *Thermomyces lanuginosus* lipase activity, allows achieving a good fit between experimental data and simulation results.
2. According to the results of study, experimental data exhibit two distinct types of substrate (FPONDS) concentration decay: one exponential (in respect to time) and the other of t^{-1}-type. This indicates that, in the *dU/dt* differential equation in system Eq (3.4), first and second order (in respect to the substrate concentration) terms are competing and should be taken into account in numeric modeling.
3. Numeric simulations have revealed that a good fitting might be obtained only taking into account non-linear substrate wash off process, which could be explained in a simplified way as a complex outcome of two different linear wash off rates: one for the electrode surface/substrate boundary (stronger bond, lower wash off rate) and the other (weaker attraction, much higher wash off rate) for the substrate/substrate layer boundary. In this model of interface, it is assumed that substrate forms only very few substrate/substrate boundaries (pseudo-multilayer interfacial structure), thus wash off rate could be seen as linearly (with respect to the substrate concentration) dropping from high value for substrate/substrate boundary, down to low value for electrode surface/substrate boundary, therefore the whole process then becomes second order with respect to the substrate concentration.

4 Computer modeling of biosensor with controllable permeability membrane

4.1 Introduction

The action of the biosensor is determined by a number of the parameters attributed to the: (i) biological system, such as the catalytical capacity of the biosensor, the rate of the bounding of the substrate, rate of the conversion of the substrate; (ii) the transducer, such as the rate of the conversion of the product of the enzymatic reaction, the rate of the regeneration of the electrochemical system; (iii) diffusion parameters and the rates of the substrate diffusion into active center, the rate of diffusion of the product to the electrochemical system, the diffusion of the products of electrochemical conversion [Turner87]. All these mentioned parameters are constant during the considerable time of biosensor action. The slowest process is identified as a rate limiting step and determines the parameters of the biosensor. The electrochemical reactions are usually very fast compared to the enzymatic process and diffusion parameters. Thereby, there are two groups of parameters, responsible for biosensor action: biocatalytic - determined by the parameters of enzymatic conversion, and diffusion - determined by the construction of the biosensor. On the basis of these two groups of parameters two limiting biosensor action modes were identified. The first one – the kinetic mode is realized when the activity of the enzymatic conversion of the substrate is very low in comparison with the diffusion parameters. In this case all parameters of the biosensor are determined by the parameters of the catalytic process. It means, that the pH dependence of the biosensor response will be the same as the pH dependence of the enzyme activity, and the temperature dependence of the response will be determined by activation energy of the catalytic process (usually about 10 %/deg.) etc. The linearity of the biosensor response (linear dependence of the biosensor response to the substrate concentration) is usually

determined by the value of K_M and is very short. The sensitivity of the response (response to concentration ratio) is quite high.

Another limiting mode – the diffusion mode, is realized when the catalytic activity of the biosensor is very high and the slowest step is a substrate diffusion to the active center of enzyme. At this mode the parameters of the biosensor are determined by the diffusion parameters. If the pH change does not affect diffusion parameters, it means that biosensor response is insensitive to the pH fluctuations in the bulk. The influence of the temperature on the diffusion is much lower (about 2-3 %/deg.). The sensitivity of the biosensor is low. When the biosensor operates in the diffusion mode, a long linear calibration curve of the biosensor can be expected. It is a good feature, because biosensor can operate at high concentrations of the substrate. For example, linearity of glucose biosensors, designed on glucose oxidase [Laurin89] or PQQ glucose dehydrogenase [Laurin04] usually reach only few mM. Only in some cases it can be extended up to 15 mM [Laurin04, Kanap92]. This can be achieved by switching biosensor action into deep diffusion mode, or artificially lowering the concentration of the substrate on the outer surface of the outer membrane. These modes of the biosensor action were described in a (large) number of papers [Mell75, Kulys86, Schul97, Baron02, Baron03, Baron04].

The possibility of switching the modes of action opens up a nice opportunity to manage analytical parameters of the biosensor. Sometimes it is useful, especially, when the biosensor operates in a system where the substrate concentration varies on large scale.

The goal of this paper is the mathematical modeling of the biosensor action, where the catalytic capacity of the biosensor is affected by the pH, and when the diffusion parameters of the membrane can be regulated. The response time and the linearity of the biosensor will also be analyzed.

4.2 Physical model

The flat biosensor with the enzyme layer deposited on the flat electrode and covered with the flat membrane has been investigated. A number of

electrochemical biosensors have such construction. Even if the outer membrane is omitted, the thickness of enzymatic layer on the surface of the electrode and non- mixing solvent layers act like the outer membrane. It is assumed that the thickness of the enzyme layer is *c* and stable during all procedure. Flat porous membrane of thickness δ=d-c possess flexible thickness and permeability characterized by diffusion coefficients for substrate and reaction product $D_{SM} = D_{PM} = D_{membr.}$. The enzyme activity and the membrane permeability are dependent on the pH. Currently modeled system is schematically presented in Fig. 4.1.

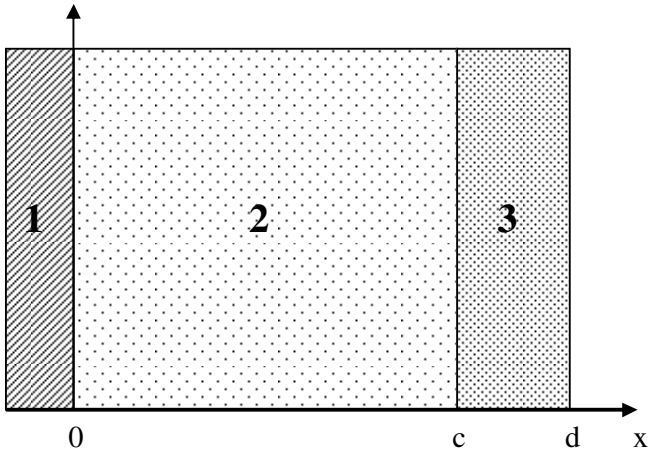


Fig. 4.1. Cross-section scheme of the model used in the present study: 1 – electrode, 2 – enzyme layer, 3 – membrane.

4.3 Mathematical model

It is assumed the classical scheme, where enzyme (E) converts substrate (S) into reaction product (P):

$$S \xrightarrow{E} P \tag{4.1}$$

Such a biosensor mathematical model could be described by using two dimensional reaction–diffusion equations containing a nonlinear term related to the Michaelis–Menten kinetics with the reaction product inhibition. In this case, it is additionally assumed that the diffusion coefficients and the enzyme

activity are dependent on the pH and the temperature. Equations governing the processes occurring in area 2 (Fig. 4.1) are [Frey07]:

$$\begin{cases} \dfrac{\partial S}{\partial t} = D_{SE}(T)\dfrac{\partial^2 S}{\partial x^2} - \dfrac{V_{Max}(pH,T)\cdot S}{K_M\cdot(1+P/K_P)+S} \\ \dfrac{\partial P}{\partial t} = D_{PE}(T)\dfrac{\partial^2 P}{\partial x^2} + \dfrac{V_{Max}(pH,T)\cdot S}{K_M\cdot(1+P/K_P)+S} \end{cases}, \; x\in(0,c), \tag{4.2}$$

Equations describing the processes occurring in area 3 are:

$$\begin{cases} \dfrac{\partial S}{\partial t} = D_{SM}(pH,T)\dfrac{\partial^2 S}{\partial x^2} \\ \dfrac{\partial P}{\partial t} = D_{PM}(pH,T)\dfrac{\partial^2 P}{\partial x^2} \end{cases}, \; x\in(c,d), \tag{4.3}$$

where symbols in italics are S – substrate concentration, D_{SE}, D_{PE} – substrate and reaction product diffusion coefficients inside area 2, D_{SM}, D_{PM} – substrate and reaction product diffusion coefficients inside membrane (area 3), V_{Max} – maximum enzymatic rate, Km – Michaelis constant, K_P – reaction product inhibition constant, T – temperature, pH – pH inside membrane.

The maximum enzymatic rate dependence on the pH is modeled using the Gauss function with the center pH_V=6 and the dependency on temperature is modeled by the linear function with 10%/degree rate and the center at 20°C:

$$V_{Max}(pH,T) = V_{Max0}\cdot\frac{T-10}{10}\cdot e^{-(pH-6)^2} \tag{4.4}$$

where V_{Max0} – a typical maximum enzymatic rate at 20°C and the pH = 6. The diffusion coefficients inside the enzymatic layer (area 2) are modeled using the linear function with 3%/degree rate and the center at 20°C:

$$\begin{cases} D_{SE}(pH) = D_{SE0}\cdot\dfrac{T+13}{33} \\ D_{PE}(pH) = D_{PE0}\cdot\dfrac{T+13}{33} \end{cases} \tag{4.5}$$

where D_{SE0} and D_{PE0} – the substrate and the reaction product diffusion coefficient inside the enzymatic layer (area 2) at 20°C temperature. The

diffusion coefficients dependency on time inside membranes modeled using several different models – Gaussian, linear and constant:

$$\begin{cases} D_{SM}(pH,T) = D_{SE}(T)\cdot e^{-(pH-pH_M)^2} \\ D_{PM}(pH,T) = D_{PE}(T)\cdot e^{-(pH-pH_M)^2} \end{cases}, \tag{4.6}$$

or

$$\begin{cases} D_{SM}(pH,T) = D_{SE}(T)\cdot(0.2\cdot pH - 0.7) \\ D_{PM}(pH,T) = D_{PE}(T)\cdot(0.2\cdot pH - 0.7) \end{cases}, \tag{4.7}$$

or

$$\begin{cases} D_{SM}(pH,T) = D_{SE}(T)\cdot 0.2 \\ D_{PM}(pH,T) = D_{PE}(T)\cdot 0.2 \end{cases}. \tag{4.8}$$

Initial conditions:

$$S(0,x) = 0, \text{ kai } x<d;\ S(0,d) = S_0;\ P(0,x) = 0. \tag{4.9}$$

Boundary conditions:

$$\begin{aligned} &\frac{\partial S}{\partial x}(t,0) = 0, S(t,d) = S_0,\ P(t,0) = 0, P(t,d) = 0, \\ &D_{SE}(T)\frac{\partial S}{\partial x}(t,c-0) = D_{SM}(pH,T)\frac{\partial S}{\partial x}(t,c+0), \\ &D_{PE}(T)\frac{\partial P}{\partial x}(t,c-0) = D_{PM}(pH,T)\frac{\partial P}{\partial x}(t,c+0). \end{aligned} \tag{4.10}$$

The observed sensor characteristics:

Reaction product gradient (proportional to sensor amperometric response) i: $i(pH,T,S_0,t) = \left.\frac{\partial P}{\partial x}\right|_{x=0}$ (4.11)

Sensor steady state response: $i_{fin}(pH,T,S_0)$. (4.12)

Sensor steady state achievement time t_{fin}:

$$t_{fin}(pH,T,S_0) = \max\{t : i(pH,T,S_0,t) < 0.95\cdot i_{fin}(pH,T,S_0)\}. \tag{4.13}$$

Sensor linear range S_{linear_range}:

$$S_{linear\_range} = \max\left\{S_0 : 0.95 < \frac{i_{fin}(pH,T,S_0)S_{0\_initial}}{i_{fin}(pH,T,S_{0\_initial})S_0} < 1.05\right\}. \tag{4.14}$$

Sensor sensitivity B: (4.15)

$$B = \frac{i(pH,T,S_0,t_2) - i(pH,T,S_0,t_1)}{S_0 \cdot (t_2 - t_1)},$$
$$t_2 = \max\{t : i(pH,T,S_0,t) < 0.5 \cdot i_{fin}(pH,T,S_0)\},.$$
$$t_1 = \max\{t : i(pH,T,S_0,t) < 0.3 \cdot i_{fin}(pH,T,S_0)\}.$$

Model described above assumes that oxidation-reduction reaction on electrode is instantaneous. This is true when membrane permeability is changed before experiment and remaint constant during experiment and electrode remains active for whole experiment. In case, when electrode is switched on only after some time from start of experiment, electrode electrochemical reaction rate should be taken into account. This method is known as electrochemical stripping. Such technique could be improved by combining it with controllable permeability membrane, i.e. by switch membrane on/off only at some specially selected moment of time. Such biosensor modeling requires model (4.1) update with electrode reaction:

$$S \xrightarrow{E} P, \tag{4.16}$$
$$P \xrightarrow{k_e} P^*, \tag{4.17}$$

where P^* - converted reaction product, which is formed by oxidation/reduction of rection product on electrode.

Reaction (4.17) happens only on electrode surface, so it should be included only into boundary condition.

$$\begin{aligned}
&\frac{\partial S}{\partial x}(t,0) = 0, S(t,d) = S_0, \\
&\frac{\partial P}{\partial t}(t,0) = D_{PE}(T)\frac{\partial P^2(t,0)}{\partial x^2} - k_e(t)c_k P(t,0), P(t,d) = 0, \\
&D_{SE}(T)\frac{\partial S}{\partial x}(t,c-0) = D_{SM}(pH,T)\frac{\partial S}{\partial x}(t,c+0), \\
&D_{PE}(T)\frac{\partial P}{\partial x}(t,c-0) = D_{PM}(pH,T)\frac{\partial P}{\partial x}(t,c+0).
\end{aligned} \tag{4.18}$$

where $k_e(t)$ is electrochemical reaction rate on electrode, c_k – dimension settlement constant.

The observed sensor characteristics:

Reaction product conversion rate is proportional to the generated biosensor current i: $i(pH,T,S_0,t) = k_e(t)P(t,0)$. (4.19)

Maximum level of sensor response:

$$i_{\max}(pH,T,S_0) = \max\{i(pH,T,S_0,t)\}. \quad (4.20)$$

4.4 Computer simulation setup and results

Semi universal software package was created to automate biosensor computer modeling. This software package was created in Matlab environment. Software package features possibility to change many parameters of biosensor at same time, also it is taking into account membrane permeability and enzyme activity dependence on temperature and medium pH, also it allows to control time moment when electrode or membrane is witched on. The linear part of (4.2)-(4.2) equation system was approximated and solved using Crank-Nicolson finite differences scheme [Samar01]. The non-linear part of the system was handled using a simple iteration method.

A series of computational simulations using software package described above were performed to investigate how electrode readings would differ if this amperometric biosensor worked under the presented model when: I) the pH, the temperature and the membrane diffusion coefficients are constant, but the maximum enzymatic rate V_{max} is monotonously increased, the simulation targets are sensitivity B eq. (4.15) and the linear range S_{linear_range} eq. (4.14); II) the pH, the temperature and the maximum enzymatic rate V_{max} are constant, but the membrane diffusion coefficient is monotonously increased up to the diffusion coefficients equal to the ones in area 2, the simulation targets are the same as for exp. I); III) the pH and temperature are increased linearly through the range, V_{max} is calculated according to eq. (4.4), the 2nd area diffusion coefficients are calculated according to eq. (4.5), the membrane diffusion coefficients are calculated according to eq. (4.6), $pH_M > 6$, the simulation targets are the linear range S_{linear_range} eq. (4.14) and the sensor steady state achievement time t_{fin} eq. (4.13); IV) the same as III), but $pH_M < 6$; V) the same

as III), but pH_M=6; VI) the same as III, but the membrane diffusion coefficients are constant with regard to pH, eq. (4.8); VII) the same as III, but the membrane diffusion coefficients are calculated according to linear equation system (4.7).

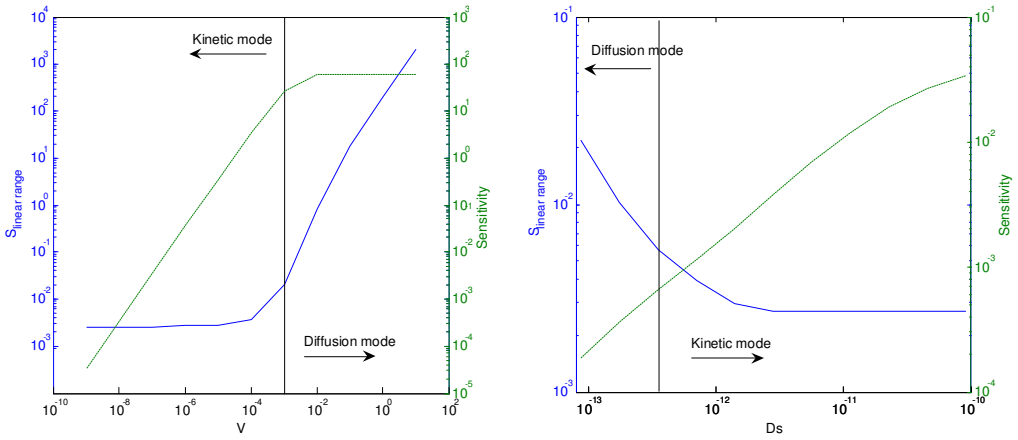
The following values were used in simulation for experiment I): S_0 =1.46×10^{-6} mol m^{-3} (for sensitivity measurement and for linear range test substrate concentration by 10% with each iteration, while condition (4.14) is matched), $S_{0\_initial}$ =1×10^{-6} mol m^{-3}, $D_{SE} = D_{PE} = D_{SM} = D_{PM}$ = 0.9×10^{-10} mol m^{-3}, V_{Max} = [10^{-9}..10] mol m^{-3} s^{-1}, Km = 10^{-1} mol m^{-3}, K_P = 10^{19} mol m^{-3} (value big enough to suppress the effect of inhibition for this experiment), T = 20°C, pH = 6, sensor thickness (including membrane) d = 2×10^{-4} m, sensor enzymatic layer thickness c = 1.6×10^{-4} m, integration period t_{int} = 15000 sec. The values used for experiment II) were the same except: V_{Max}= 1×10^{-6} mol m^{-3} s^{-1}, $D_{SM} = D_{PM}$ = [8.79×10^{-9}.. 0.9×10^{-10}] mol m^{-3}. The values used in experiment III) were the same as for I) except, S_0 =2.62×10^{-1} mol m^{-3} (for steady state time measurement), D_{SM} ,D_{PM} calculated according to eq. (4.6), pH_M = 7, V_{Max} = 1×10^{-4} mol m^{-3} s^{-1}, T = [15..25]°C, pH = [4..8], K_P = 1×10^{-4} mol m^{-3}. The values used in experiment IV) were the same as for experiment III) except pH_M = 5. The values used in experiment V) were the same as for experiment III) except pH_M = 6. The values used in experiment VI) were the same as for experiment III) except that the membrane diffusion coefficients substrate and the reaction product were calculated according to eq. (4.8). The values used in experiment VII) were the same as for experiment III) except that the membrane diffusion coefficients substrate and the reaction product were calculated according to linear equation system (4.7).

Using a combination of biosensor parameters and numerical simulation, biosensor action was extended into kinetic and diffusion modes. As a measure of the biosensor action, the linearity of the biosensor response was calculated. As a criterion of the linearity the limit concentration of the substrate when the biosensor response curve differs more than 5 % from the hypothetic linear dependence was considered. The biosensor response time was also considered

as one of the important parameters. The response time was calculated as a time when 95% of the steady state signal is achieved.

In the Fig. 4.2 the typical parameters of the biosensor, operating in the kinetic and diffusion modes are presented. At the stable diffusion parameters (Fig. 4.2A) and low activity of the biocatalytic layer, the limiting process is an enzymatic conversion of the substrate, thereby, the concentration of the substrate inside enzymatic layer and in the bulk will be the same. If to take into account, that K_M of the enzyme is stable, then the linearity of the biosensor is stable and it is estimated about the third part from K_M value. The sensitivity of the biosensor (taking into account only linear part of the curve) increases with the increase of the activity of the enzyme.

At the high activity of the enzyme, the limiting step becomes the diffusion of the substrate through the enzymatic membrane. In this case the sensitivity of the biosensor depends only on the substrate supply rate. At the stabile substrate concentration, the sensitivity of the biosensor is also stable. In the diffusion mode of action the actual concentration of the substrate close to the active center of the enzyme will be much lower compared with the substrate concentration in the bulk. This difference of the substrate concentrations will increase increasing of the enzyme activity; thereby, the sensitivity of the biosensor will increase with increasing the activity of the biocatalytic layer.



(A) **(B)**

Fig. 4.2. A. Dependence of the: sensor response linear range (left axis), and sensitivity (right axis) on maximum enzymatic rate. Diffusion coefficient of the outer membrane DSM = DPM = 0.9×10-10 mol m-3. Substrate concentration S0 =1.46×10-6 mol m-3 (for sensitivity measurement).
B. Dependence of the: sensor response linear range (left axis) and sensitivity (right axis) on outer membrane diffusion coefficient. VMax= 1×10-6 mol m-3 s-1.

Legend: blue (solid) – linear response range, green (dashed) – sensitivity.

Analogous data can be observed when the sensor operates with a variable permeability of the outer membrane (Fig. 4.2B). At high value of the D_S, the permeability of the membrane is high; thereby the limiting step is the activity of the enzyme. At stable $V_{max.}$ and K_M, both, linear response range of the biosensor and the sensitivity are stable. At lower diffusion coefficient the biosensor switches to the diffusion mode of action. It leads to the decrease of the biosensor sensitivity, because the substrate concentration in the enzymatic layer will be lower than in the bulk. This difference in the concentrations will increase with decrease of the diffusion coefficient and it will reflect limited substrate capability to reach the active center of the enzyme. Thereby, the sensitivity of the biosensor will decrease, and linearity (linear response range) will increase, as it can be seen in Fig. 4.2B.

These data indicate that the selected parameters of the biosensor action are correct and they can be applied to further calculations. The curves obtained on the basis of the mathematical simulations are close to the experimental data obtained in experiments published by [Laurin89] where the behavior of the electrochemical biosensors with outer membranes possessing different

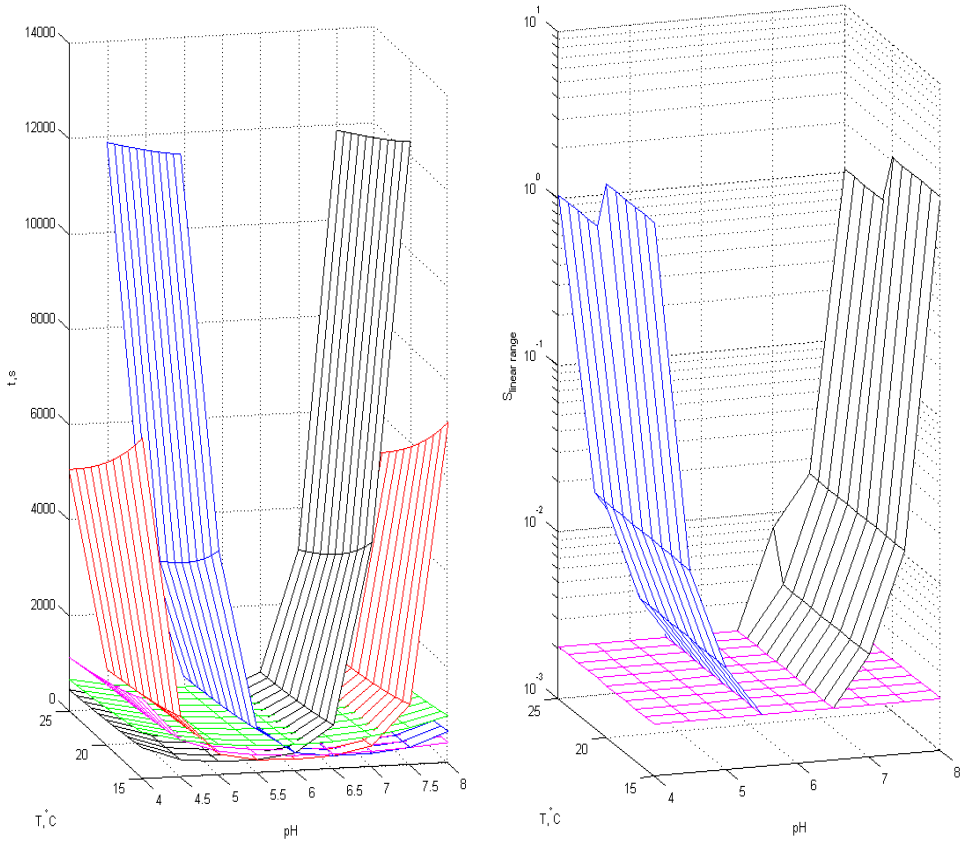
permeability has been investigated. A number of membranes with different permeability have been obtained by acetylating of the cellulose membrane.

The activity of the enzyme is decreasing in time, and the outer membrane can be glued with outside proteins, lipids, cells, etc. This often occurs during a long time of exploitation of the biosensor. Lets consider the case when the permeability of the membrane or the activity of the enzyme, or both could be managed in the already prepared biosensor. This feature can be very useful when there is a need to control the activity of the biosensor, or to use the biosensor as a switcher. A very suitable instrument for this purpose can be the pH factor. A number of artificial membranes possess different permeability to the substrate with the pH. There can be several reasons of such behavior. Some membranes (especially of the protein nature) have ionogenic groups, which can be responsible for the charge of the membrane. The charge can influence the shrinking of the membrane, thereby, the permeability and thickness can be regulated by the pH. On the other hand, the charged substrate diffusion capability through the charged membrane can also be regulated by the pH. Several cases, including the different enzyme activity and membrane permeability, and sensitivity to the temperature changes, have been modeled. The results of the biosensor action are depicted in Figure 3. The parameters of the biosensors have been selected so that in the case when the permeability of the membrane does not depend on the pH, the biosensor will operate in the kinetic mode. However, at high and low pH due to low activity of the enzyme, the biosensor action will be switched into the diffusion mode. The response time of the biosensor is mostly sensitive to this switch, and curve 5 of Fig. 4.3 A clearly indicates the boundary regions of both modes of action. The linear range of the biosensor action expressed in the logarithm scale (Fig. 4.3B) is not the best way to visualize boundary regions; however it is a good method for the analysis of the deep diffusion mode.

Usually a membrane does not possess strongly expressed pH optima (like enzyme), and change its permeability in the wide region of the pH. Suppose, the permeability of the membrane depends linearly on the pH, is in the interval

pH 4-8 and increases 9 times. It is a typical case for membranes that can shrink on the pH. (Fig. 4.3 A, curve 4) At high pH (pH 8) the permeability of the membrane is high, however the activity of the enzyme is very low (pH optima at 6) and the rate of the substrate supply is almost at the same value as the substrate consumption that leads to the kinetic or boundary mode of action. At pH 6 the activity of the enzyme is top high and the biosensor operates in the deep kinetic mode possessing relatively fast signal and a short linear range (Fig. 4.3B) At a lower pH both the enzyme activity and the membrane permeability are low and the biosensor operates again in the boundary or diffusion mode of the action.

Suppose the permeability of the membrane depends on the pH in the same manner as the activity of the enzyme. Such a situation can be observed when both the charge of the membrane and the charge of the substrate (or product) depend on the pH. Let both enzyme activity and membrane permeability dependency on pH be of the same Gaussian manner. Lets consider the situation when the pH optima of the membrane and the enzyme are the same, i.e. equal to pH 6. It means that the isoelectric point of the substrate and the membrane are the same – pH 6. In this case a well expressed diffusion regime of the action is observed (that indicates the increased time of the response (Fig. 4.3A, curve 3)) at low and high pH, and quite a wide region (around pH 6), where the biosensor operates in the kinetic mode of action.



(A) **(B)**

Fig. 4.3. (A) Dependence of sensor response time on temperature (T) and pH. (B). Dependence of sensor linear range length (logarithmic scale) on temperature and pH.
pH optima of the enzyme pHV = 6.
1 (blue) - membrane permeability depends on pH with pH optima at 7 (pHM = 7, eq. 4.6);
2 (black) - membrane permeability depends on pH with pH optima at 5 (pHM = 5, eq. 4.6);
3 (red) - membrane permeability depends on pH with pH optima at 6 (pHM = 6, eq. 4.6);
4 (magenta) - membrane permeability linearly depends on pH (DSM, DPM increases 9 times in the region from pH 4 to pH 8, eq. 4.7);
5 (green) - membrane permeability does not depend on pH (eq. 4.8).

However, the diffusion regime is not very deep, and this could be seen from the negligible increase of the linearity of the biosensor action (Fig. 4.3 B, curves 3-5).

If the pH optima of the enzyme and the membrane are different, a tremendous difference is observed on the biosensor action. When the pH optima of the permeability of the membrane is higher than the enzyme activity optima (Fig. 4.3, curve 1), the maximal permeability of the membrane is shifted to the region with a higher pH. The kinetic mode of the action is also

shifted. In Fig. 4.3 only the left wing of the curve is visible. If the pH optimum of the permeability of the membrane is lower than the enzyme (Fig. 4.3, curve 2), the same picture is observed, but it is shifted into the region with lower pH, and only the right wings of the curves are visible in Fig. 4.3. Getting deeper into the diffusion mode of the action of the biosensor, the response time is increasing several times, however the linearity of the biosensor is increasing by several magnitudes.

When biosensor operates in electrochemical stripping mode then several magnitudes higher response signal level might be observed for short period of time. Typical such sensor response is shown in Fig. 4.4, assuming that electrode is witched on after 500 seconds after start of experiment.

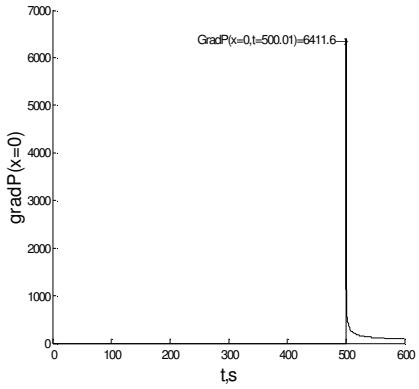


Fig. 4.4. Biosensor response when sensor operates in electrochemical stripping mode and electrode is switched on at *t*=500 sec, mebrane is switched off for whole experiment.

Switching membrane on at the beginning of experiment actually lowers response level. But making this switch some time later (i.e. *t*=60 sec.) allows to obtain much higher biosensor response. Details provided in Fig. 4.5.

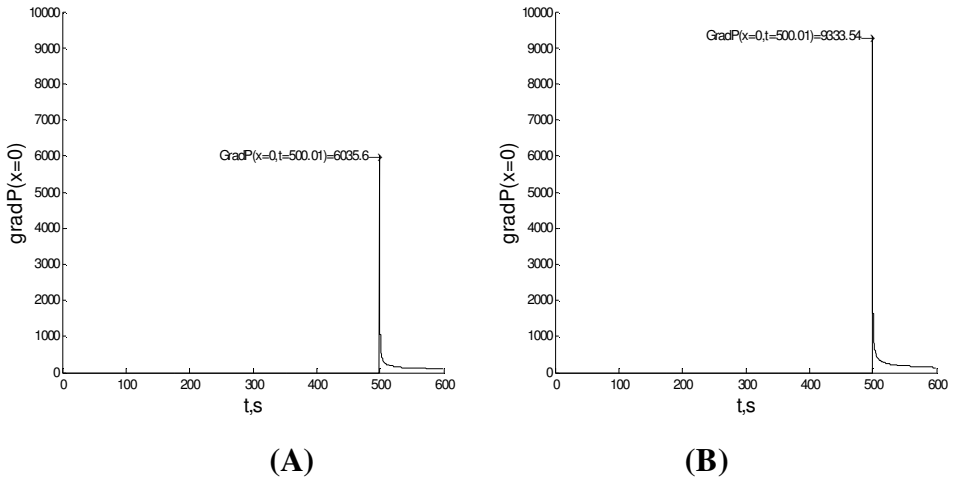


Fig. 4.5. Biosensor response when sensor operates in electrochemical stripping mode and electrode is switched on at *t*=500 sec, mebrane is switched on at A) *t*=0 sec., B) *t*=62 sec.

Making this switch later increases response signal level even a bit more, but this is true until some moment (specific for each biosensor setup) is reached. Making membrane switch even later causes biosensor response signal level degradation. Experiment results described above were obtained with absence of reaction inhibition by reaction product. This isn't true in all cases. Second set of computer simulations was performed with inhibition constant $K_P = 10^{-4}$ mol m^{-3}. These experiments still showed some superiority of controllable membrane over static membrane, but to much lesser degree.

It is to be hoped that these observed dependencies will be useful for the creation of the biosensor monitoring systems.

4.5 Conclusions

1. Proposed method of the mathematical modeling of the action modes of the electrochemical biosensors with an outer controllable diffusion membrane allowed to identify what biosensor parameters show when sensor switches between modes of operation.
2. Analysis of few specific cases, when enzyme activity and membrane permeability depend on, revealed that biosensor response time in highly sensitive to the mode of operation of biosensor, especially in boundary cases.

3. Setting biosensor to deep diffusion mode allows achieving of biosensor linear range expansion by several magnitudes.
4. Sensor could be easily switched to deep diffusion mode when membrane permeability nonlinearly depends on medium pH and permeability maximum is different from enzyme activity maximum.
5. Proposed and analyzed biosensor operating with controllable in time permeability membrane yields higher response signal level compared to biosensor with static membrane, when both biosensors are working in electrochemical stripping regime, membrane of the first biosensor is turned on at the selected moment of time, enzymatic reaction inhibition by reaction product is low.

5 Conclusions

Basing on the results of the computer modeling of three biosensors with innovative structure the following main conclusions were made:

1. Model described by [Verger72] cannot be applied directly for *Thermomyces lanuginosus* lipase activity assessing biosensor. Constant substrate concentration cannot be assumed thus model should be extended with substrate kinetic equation. Such model extension allows obtaining good fit between experimental and computer simulation results. This computer modeling additionally allows to evaluate enzymatic reaction rate constant.
2. According to the results of lipase activity assessment biosensor (with substrate covered electrode) study, experimental data exhibit two distinct types of substrate concentration decay: one exponential (in respect to time) and the other of t^{-1}-type. This indicates that, in the *dU/dt* differential equation in system Eq (3.4), first and second order (in respect to the substrate concentration) terms are competing and should be taken into account in numeric modeling. Good fit between experimental and computer simulation results could be obtained when both terms are taken into account.
3. Biosensor with controllable permeability membrane could be easily switched to deep diffusion mode when membrane permeability nonlinearly depends on medium pH and permeability maximum is different from enzyme activity maximum.
4. Biosensor operating with controllable in time permeability membrane yields higher response signal level compared to biosensor with static membrane, when both biosensors are working in electrochemical stripping regime, membrane of the first biosensor is turned on at the

selected moment of time, enzymatic reaction inhibition by reaction product is low.

6 Rerefences

[Bard01] A. J. Bard, L. R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, second ed., Wiley, New York, 2001.

[Baron02] R. Baronas, F. Ivanauskas, J. Kulys, Modelling dynamics of amperometric biosensors in batch and flow injection analysis. *J. Math. Chem.* 32, 225-237, 2002.

[Baron03] R. Baronas, F. Ivanauskas, J. Kulys, and M. Sapagovas, Modelling of amperometric biosensors with rough surface of the enzyme membrane. *J. Math. Chem.*, 34, 227, 2003.

[Baron04] R. Baronas, J. Kulys, F. Ivanauskas, Modelling amperometric enzyme electrode with substrate cyclic conversion. *Biosens. Bioelectron.*, 19, 915, 2004.

[Beiss00] F. Beisson, A. Tiss, C. Riviere, R. Verger, Methods for lipase detection and assay: a critical review. *Eur. J. Lipid Sci. Technol.*, 133-153, 2000.

[Blum91] L. J. Blum, P. R. Coulet, *Biosensor Principles and Applications*, CRC Press, 1991.

[Born99] U.T. Bornscheuerand, R.J. Kazlauskas, *Hydrolases in Organic Synthesis Regio- and Stereoselective Biotransformations*, Wiley-VCH: Weinheim, 1999.

[Coop04] J. Cooper, A. E. G. Cass, *Biosensors: A Practical Approach*, Oxford University Press, 2004.

[Frey07] P. A. Frey, A. D. Hegeman, *Enzymatic Reaction Mechanisms,* Oxford University Press, Oxford, 2007.

[Houde04] A. Houde, A. Kademi, D. Leblanc, Lipases and their industrial applications, *Appl. Biochem. Biotechnol.*, 118(1–3), pp. 155–170, 2004.

[Ignat05] I. Ignatjev, G. Valinčius, I. Švedaitė, E. Gaidamauskas, M. Kažemėkaitė, V. Razumas, A. Svendsen, Direct amperometric determination of lipase activity, *Anal. Biochem.*, 344(2), pp. 275–277, 2005.

[Kanap92] J.J. Kanapienene, A.A. Dedinaite and V. Laurinavicius, *Sens. & Actuators B*. 10, 37, 1992.

[Kulys86] J. Kulys, V.V. Sorochinski and R.A. Vidziunaite, Transient response of bienzyme electrodes. *Biosensors,* 2, 135, 1986.

[Laurin04] V. Laurinavicius, J. Razumiene, A. Ramanavicius, A.D. Ryabov, *Biosens. Bioelectron*. 20, 1217, 2004.

[Laurin89] V.A. Laurinavicius, J.J. Kulys, V.V. Gureviciene and K.J. Simonavicius, *Biomed. Biochim. Acta*. 48, 905, 1989.

[Mell75] L.D. Mell and J.T. Maloy, A model for the amperometric enzyme electrode obtained through digital simulation and applied to the glucose oxidase system. *Anal. Chem.*, 47, 299, 1975.

[Puida07] M. Puida, F. Ivanauskas, I. Ignatjev, G. Valinčius, V. Razumas, Computational modeling of the amperometric bioanalytical system for lipase activity assay: a time-dependent response. *Nonlinear Analysis: Modelling and Control*, 12 (3), 245–251, 2007.

[Puida08] M. Puida, F. Ivanauskas, I. Ignatjev, G. Valincius, V. Razumas, Computational modeling of the electrochemical system of lipase activity detection. *Sensors*, 8 (6), 2008.

[Samar01] A. A. Samarskii, *The Theory of Difference Schemes*, Marcel

Dekker, New York-Basel, 2001.

[Schell92] F. Scheller, F. Schubert, *Biosensors*, Elsevier, 1992.

[Schmi98] R. D. Schmid, R. Verger, Lipases: Interfacial enzymes with attractive applications, *Angew. Chem. Int. Ed.*, 37(12), pp. 1608–1633, 1998.

[Schul97] T. Schulmeister, J. Rose and F. Scheller, Mathematical modelling of exponential amplification in membrane-based enzyme sensors. *Biosens. Bioelectron.* 12, 1021, 1997.

[Shimi88] T. Shimidzu, A. Ohtani, K. Honda, Charge-previous termcontrollablenext term poly pyrrole/poly electrolyte composite previous termmembranes.next term Part III. Electrochemical deionization system constructed by anion-exchangeable and cation-exchangeable polypyrrole electrodes, J. *Electroanal. Chem.,* 251, 1988.

[Tran93] M. C. Tran, S. A. Jackson, *Biosensors*, Springer, 1993.

[Turner87] A.P.F. Turner, I. Karube and G.S. Wilson, *Biosensors: Fundamentals and Applications,* Oxford University Press, Oxford, 1987.

[Valin05] G. Valincius, I. Ignatjev, G. Niaura, M. Kažemėkaitė, Z. Talakaitė, V. Razumas, A. Svendsen, Electrochemical method for the detection of lipase activity. *Anal. Chem.* 2005, 77 (8), 2632-2636.

[Verger72] R. Verger, M. C. E. Mieras, G. H. De Haas, Action of phospholipase A at interfaces, *J. Biol. Sci.*, 248(11), pp. 4023–4034, 1972.R. Verger, M. C. E. Mieras, G. H. De Haas, Action of phospholipase A at interfaces, *J. Biol. Sci.*, 248(11), pp. 4023–4034, 1972.

[Yang06] G. Z. Yang, M. Yacoub, *Body Sensor Networks*, Birkhäuser,

2006

[Yu06] B. Yu, N. Long, Y. Moussy, F. Moussy, A long-term flexible minimally-invasive implantable glucose biosensor based on an epoxy-enhanced polyurethane membrane, *Biosensors and Bioelectronics*, 21 (12), 2006.

List of publications

1. M. Puida, F. Ivanauskas, I. Ignatjev, G. Valinčius, V. Razumas, Computational modeling of the amperometric bioanalytical system for lipase activity assay: a time-dependent response. *Nonlinear Analysis: Modelling and Control*, 12 (3), p. 245–251, 2007.
2. M. Puida, F. Ivanauskas, I. Ignatjev, G. Valincius, V. Razumas, Computational modeling of the electrochemical system of lipase activity detection, *Sensors*, 8 (6), p. 3873-3879, 2008.
3. M. Puida, F. Ivanauskas, V. Laurinavicius, Mathematical modeling of the action of biosensor possessing variable parameters, (accepted for publishing in *Journal of Mathematical Chemistry* on 17th April, 2009).

Curriculam vitae

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Reziumė

Kompiuterinis modeliavimas yra svarbus įrankis, kuriant naujus ir tobulinant esamus biojutiklius. Tai tyrimo metodas, padedantis greitai ir efektyviai ištirti, kokiomis savybėmis pasižymės naujai sukurti biojutikliai, kokie procesai vyks juose ir kokie parametrai labiausiai lems šių jutiklių atsaką. Šis metodas, sumažindamas fizinių eksperimentų poreikį iki minimumo, padeda taupyti fizinius ir žmogiškuosius resursus. Biojutikliai yra jautrūs, maži ir greiti analitiniai prietaisai, kasdien vis plačiau taikomi tokiose srityse, kaip sveikatos priežiūra, aplinkosauga, maisto, popieriaus, tirpiklių pramonė. Struktūrinės ir kitos šių jutiklių inovacijos padeda plėsti jų taikymo sritis bei gerinti jų analitines savybes. Kompiuterinis modeliavimas yra priemonė, kuri padeda iš anksti nuspėti, koks bus biojutiklio atsakas konkrečiomis aplinkos sąlygomis, koks bus jo jautrumas, jautrumo ribos ar greitaveika, kitaip tariant, padeda numatyti kiek praktiška būtų šio jutiklio gamyba.

Šiame darbe nagrinėjami trys struktūriniu požiūriu inovatyvūs biojutikliai: lipazės aktyvumo nustatymo biojutiklis su substratu, ištirpintu micelėse, lipazės aktyvumo nustatymo biojutiklis su substratu, padengtu elektrodu bei teorinis biojutiklis su valdomo laidumo (pavyzdžiui, keičiant terpės pH) membrana. Šio darbo tikslai buvo parinkti tinkamus matematinius modelius šių jutiklių kompiuteriniam modeliavimui. Taikant kompiuterinį modeliavimą, siekta ištirti koks bus šių biojutiklių atsakas tam tikromis aplinkos sąlygomis, kokie vidiniai bei išoriniai parametrai labiausiai lemia jų atsaką.

Šių biojutiklių tyrimui sukurta tokio tipo biojutiklių modeliavimą automatizuojantis programinės įrangos paketas veikiantis *Matlab* aplinkoje. Kompiuterinio modeliavimo būdu nustatyta, kad modeliuojant lipazės aktyvumo nustatymo biojutiklį su substratu, ištirpintu micelėse, negalima daryti prielaidos, jog viso eksperimento metu substrato koncentracija išlieka pastovi, ir modelis turi būti papildytas substrato kinetine lygtimi. Taip pat

nustatyta fermentinės reakcijos greičio konstanta. Ištirta, kad papildomo difuzinio sluoksnio įvedimas (kitus sluoksnius išlaikant tokio pat storio) sumažina pradinį sistemos atsaką, o difuzinio sluoksnio siaurinimas didina sistemos atsaką.

Kompiuterinio modeliavimo būdu nustatyta, kad klasikinis modelis tinka paaiškinti tik daliai lipazės aktyvumo nustatymo biojutiklio su substratu padengtu elektrodu eksperimentinių duomenų. Taip pat tyrimai parodė, kad papildomos tarpinės būsenos įvedimas į fermentinės reakcijos mechanizmą negali paaiškinti dalies fizinio eksperimento duomenų. Ištyrus fizinio eksperimento duomenis pseudologaritminėse bei atvirkštinėse skalėse padaryta prielaida, kad substrato kinetinėje lygtyje gali konkuruoti du nariai (pirmos ir antros eilės substrato atžvilgiu). Netiesiniu antros eilės substrato atžvilgiu nusiplovimo nariu papildžius substrato kinetinę lygtį, gautas geras fizinio ir skaitinio eksperimento duomenų sutapimas. Šis netipiškas nusiplovimo narys gali būti paaiškintas tuo, kad elektrodas yra padengtas vos keliais substrato sluoksniai, kurie susirišę tarpusavyje bei su elektrodu skirtingo stiprumo ryšiais: stipresnis ryšys - substratas-elektrodas bei silpnesnis ryšys - substratas-substratas. Tokiu atveju substrato nusiplovimo greitis būtų proporcingas substrato koncentracijai ir tiesiškai su ja mažėtų nuo didelio (kai vyrauja substratas-substratas nusiplovimas) iki mažo (kai vyrauja substratas-elektrodas nusiplovimas), o visas šis sudėtingas procesas būtų matomas kaip antros eilės nusiplovimo narys.

Kompiuterinio modeliavimo būdu nustatyta, kad biojutiklio atsako nusistovėjimo laikas itin jautrus biojutiklio darbo režimui ir gali būti naudojamas kaip indikatorius, norint nustatyti, ar jutiklis dirba kinetiniu ar difuziniu režimu. Perjungiant jutiklį į gilų difuzinį režimą, jo tiesinio atsako diapazoną galima išplėsti keliomis eilėmis. Biojutiklis su valdoma membrana, kurios laidumas netiesiškai priklauso nuo terpės pH, o jos laidumo maksimumas nesutampa su fermento aktyvumo maksimumu, lengvai gali būti perjungiama iš kinetinio režimo į difuzinį ir atvirkščiai. Jutikliui dirbant elektrocheminio stripingo režimu su laike valdoma membrana, tinkamai

parenkant membranos įjungimo laiką, galima gauti žymiai stipresnį sistemos atsaką, nei tuo atveju, jei jutiklis turėtų statinę membraną. Šis atsako sustiprinimas ryškiausias, kai reakcijos produktas silpnai inhibuoja fermentinę reakciją.