Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 1045–1052

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

A comparison between different immobilised glucoseoxidase-based electrodes

Adriana Memoli [a,*], Maria Cristina Annesini [b], Marco Mascini [c], Stefano Papale [b], Stefania Petralito [a]

[a] *Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università degli Studi di Roma 'La Sapienza', Piazzale A. Moro 5, 00185 Rome, Italy*
[b] *Dipartimento di Ingegneria Chimica, Università degli Studi di Roma 'La Sapienza', via Eudossiana 18, 00184 Rome, Italy*
[c] *Dipartimento di Chimica, Università degli Studi di Firenze, via Gino Capponi 9, 50121 Florence, Italy*

Received 12 July 2001; received in revised form 31 July 2001; accepted 3 August 2001

Abstract

Biosensors obtained by immobilising glucose oxidase 'unentrapped' and 'entrapped in liposomes', both with a classical H_2O_2 amperometric electrode and with screen-printed electrochemical sensor, were compared. Electrode response, linearity range and the influence of some parameters as phospholipid nature, temperature and measurement techniques were investigated. Experimental results showed that, while with the unentrapped enzyme the output current is linear only up to about 4 mM glucose concentration, the linearity range increases up to about 20 mM using enzyme-loaded liposomes; however the low permeability of the lipid bilayer decreases the electrode sensitivity to very low values (200 nA/M for palmitoylolelyl phosphatidylcholine liposomes). The approach with screen-printed sensors showed a better performance and gave biosensors with higher sensitivity (about 14 500 nA/mM). A mathematical model, useful to compare the behaviour of the different analytical systems and to design electrodes with the required properties, was also proposed.

Keywords: Biosensors; Liposome; Glucose oxidase; Screen-printed electrodes

1. Introduction

Many biosensors based on the glucose oxidase (GOD) activity have been described for the determination of glucose. Generally, the measurement is performed by following H_2O_2 production, according to the reaction

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconic Acid} + H_2O_2$$

by means of its electrooxidation process at a noble metal anode.

The performances of these systems are however limited by the enzyme saturation kinetics which limits the measurement of relevant glucose concentrations. Dilution procedures and/or the use of barrier membranes are usually necessary to extend the biosensor linear range for assay concentrations exceeding 10 mM.

* Corresponding author. Tel.: +39-6-49913-600; fax: +39-6-49913-133
E-mail address: adriana.memoli@uniroma1.it (A. Memoli).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00145-0

Lipid bilayers, showing a low permeability to glucose and high permeability to oxygen [1], represent a suitable diffusion limiting membrane and electrodes based on GOD entrapped in liposomes seem to be very promising tools [2,3].

Glucose disposable biosensors using GOD immobilized on screen-printed electrodes (SPE) are also reported [4,5]. These electrochemical sensors offer several advantages, including low cost, small size and a high degree of electrochemical activity deriving from their high specific area and microporous structure [6].

On these basis, aiming to increase the linearity range of the system, we brought together characteristics of liposomes and SPE performances. The behaviour of different biosensors are here discussed in terms of a simple mathematical model and of a comparison of experimental data. The paper is organized as follows. We firstly develop the mathematical model to analyse the different analytical systems, mainly referring to the effects of the enzyme entrapping in liposomes; then, we report the preliminary experimental results obtained by assembling immobilised GOD, unentrapped and entrapped in liposomes, both with a classical H_2O_2 amperometric electrode and with screen-printed electrochemical sensors. Linearity range, electrode sensitivity and influence of some parameters as phospholipid nature, temperature and measurement techniques are investigated.

2. Materials and methods

2.1. Materials

Ninety percent pure enriched soya phosphatidylcholine (PC) from Natterman Phospholipids GmbH and palmitoylolelyl phosphatidylcholine (POPC) from Chemi S.p.A. were used for vesicle preparation.

GOD (E.C.1.1.3.4 from *Aspergillus niger*, 195.000 units/g solid) and poly-L-Lysine solution (0.1% w/v) were purchased from SIGMA chemical Co, St. Louis, MO; polyethylenimide (PEI, average MW $\cong$ 2.000) solution (2.5% w/v) and Sepharose 4B were purchased from Aldrich Chemie Gmbh Steinheim, Germany and Pharmacia Biotech.AB Uppsala, Sweden respectively.

Immunodyne C membrane from Pall Corporation, Glenn Cove, NY, Isopore polycarbonate membrane 0.6 μm (Millipore) and cellulose acetate membrane were used.

Phosphate buffer solution (PBS) pH 7.0 (10^{-1} M-NaN_3 0.3%) prepared with freshly distilled and deareated water was used in all experiments.

Amperometric determinations were performed in presence of KCl 0.1 M.

2.2. Methods

2.2.1. Liposome preparation

Small unilamellar vesicles were obtained from large multilamellar vesicles (MLV) prepared by hydration of a dry lipid film (150 mg) with 5 ml of PBS containing 20 mg of GOD. MLV suspension was gently shaken for 1 h and then sonicated, under a nitrogen stream, 4 times for 10 min (with intervals of 2 min) with a Sonics Vibracell 600 apparatus equipped with an exponential microprobe. The temperature was maintained constant at 20 °C by means of a water bath.

To remove unentrapped enzyme, the liposome solution was eluted through a Sepharose 4B column, using PBS as eluent. All the vesicles were collected and their presence detected by turbidity measurements; the vesicle dispersion obtained was then diluted with the buffer to a fixed lecithin concentration (11.3 mg lipids/ml).

Preliminary studies with Phospholipids B test (Wako Chemicals Gmbh) indicated that over 95% of the initial amount of phospholipid used for film preparation was recovered in the form of liposomes.

2.2.2. Electrode A preparation

2.2.2.1. Unentrapped-GOD immobilisation. Ten microliters of a GOD solution at a fixed concentration (2 mg/100 μl) were placed onto 1 cm^2 of an Immunodyne membrane. After 2 h the membrane was washed with a glycine solution 0.5 M for 30 min and then with PBS for 15 min.

2.2.2.2. GOD-loaded liposome immobilization. One square centimeter immunodyne membrane pretreated with 2 ml of Poly-L-Lysine solution (0.1%

w/v) was dipped in a enzyme-loaded liposome suspension; after 30 min the membrane was gently washed with PBS.

In both cases the Immunodyne membrane was assembled between a polycarbonate and a cellulose acetate membrane to minimise enzyme or liposome losses during its use. The set of membranes was finally positioned over a Clark electrode with a central platinum-working electrode (Ø 0.5 mm). An outer Ag/AgCl ring as pseudo-reference was used.

2.2.3. Electrode B preparation

Disposable SPE with Ag/AgCl as reference and auxiliary electrodes, and a carbon electrode (about 3 mm of diameter) containing electro-active cobalt phthalocyanine (5%), were also used to immobilise enzyme or enzyme-loaded liposomes.

2.2.3.1. GOD immobilisation. A fixed amount (10 μl) of a 1:1 GOD 4 mg/ml and PEI 2.5% mixed solution, was placed on the SPE active surface and left to dry.

2.2.3.2. GOD-loaded liposome immobilisation. Ten microliter of 1:1 PEI 2.5% solution and 4 mg/ml enzyme-loaded liposomes were put directly on the SPE active surface and left to dry.

2.2.4. Amperometric measurements

A and B electrodes were connected to a potentiostat (Amel instruments 2059) and polarised at + 650 and + 400 mV respectively.

Experiments were carried out by dipping the assembled electrodes in 20 ml of thermostated PBS/KCl containing different glucose concentrations and current values at steady state were collected.

As far as the SPE, suitable mainly for drop-on techniques, are concerned measurements were also carried out by putting a drop of solution, at different glucose concentration, directly on the active electrode surface.

In all experiments the buffer solution containing glucose was equilibrated with atmospheric air.

3. Enzymatic biosensor model

In order to acquire information on the behaviour of different enzymatic biosensors and/or to design analytic systems with the required properties, a mathematical model can be suitable.

In this work we propose a model, described in details in Appendix A, accounting for:

(a) enzymatic reaction kinetics, that is limited by the glucose (c_g) and oxygen (c_o) concentration, according to the following equation [7]

$$v = \frac{V_{max}}{1 + k_o/c_o + k_g/c_g} \quad (1)$$

Saturation constants, k_o and k_g, depend on the enzyme source: values in the range 15–100 mM for k_g and 0.5 mM for k_o have been reported [8,9].

(b) substrate and reaction product diffusion in the enzyme layer, according to the Fick's law.

(c) substrate and reaction product partition between the enzyme layer and the aqueous environment of the sample.

(d) hydrogen peroxide oxidation at the electrode surface; the output current proportional to the hydrogen peroxide concentration gradient at the electrode surface.

In order to compare the electrode behaviours, we assume that the saturation constants are not modified by the different immobilisation procedures, the diffusional phenomena being accounted for separately.

At the steady state, the model results in a set of second-order non linear differential equations and a numerical procedure is required to obtained the dimensionless output current density (η) as a function of dimensionless glucose and oxygen concentration in the sample, ξ_g^s and ξ_o^s, and of the values of the ratio between diffusion and reaction characteristic time, ψ^2.

It is worth considering the electrode behaviour at low ψ^2 values, i.e. when the diffusion time in the enzymatic layer is much lower than the reaction characteristic time; in this case, the substrate concentration may be considered as uniform in the enzymatic layer and the set of differential equations can be solved analytically to obtain the dimensionless output current density (all the symbols are defined in details in Appendix A):

$$\eta = \frac{1}{2}\theta_h \psi^2 \frac{\xi_g^s}{\xi_g^s(1 + 1/\xi_o^s) + 1} \quad (2)$$

For sake of simplicity we assumed all the partition coefficients as equal to 1. From Eq. (2), it is evident that the electrode response is linear up to a sample glucose concentration $\xi_g^s \ll \xi_o^s/(1 + \xi_o^s)$, i.e. $c_g^s \ll k_g c_o^s/(k_o + c_o^s)$, whereas a limiting value of the current density, $\eta = \theta_h \psi^2 \xi_o^s/(1 + \xi_o^s)$, independent on the glucose concentration, is obtained for $\xi_g^s \gg \xi_o^s/(1 + \xi_o^s)$. Finally it is worth noting that the output current is proportional to V_{max}, in turn proportional to the enzyme concentration on the electrode surface. When higher enzyme concentrations, corresponding to higher ψ^2 values, are used, the output current increases, but stronger diffusional limitations occur so that the simplified approach is no longer correct and the numerical solution of the complete set of differential equations is required.

Fig. 1 reports some typical trends of the output dimensionless current density obtained from the theoretical model, for different values of the oxygen concentration and of the ratio between diffusion and reaction characteristic times, ψ^2; the other model parameters have been chosen considering that the oxygen diffusion coefficient in the enzyme layer and the hydrogen peroxide diffusion coefficient are 3–4 and 2–3 times higher than that of glucose respectively. Furthermore the dimensionless values of oxygen concentration in the sample have been chosen

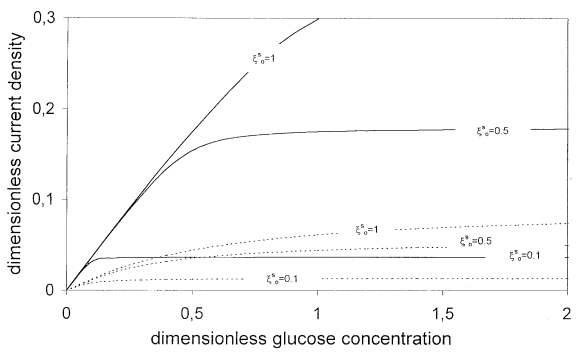

Fig. 1. Trends of output current obtained from the mathematical model for electrodes with unentrapped GOD, at different dimensionless oxygen concentration and different ψ^2 values ($\psi^2 = 10$ full lines; $\psi^2 = 1$ dotted line). Calculations have been carried out with $\theta_o = 1, \theta_h = 0.4$, $\alpha_g = \alpha_o = \alpha_h = 1$.

considering that the oxygen concentration in water at the equilibrium with atmospheric air is 0.265 mM, but may be significantly lower in samples of a different nature, as, for example, in the venous blood. As clearly shown the linear range does not extend to glucose concentration in the sample greater than k_g and is further reduced if the oxygen concentration in the sample is low. At high ψ^2 values, the linearity range is further reduced and the sensitivity to the oxygen concentration increases.

A substantial improvement of the electrode performance can be obtained if a diffusion barrier, like liposome, reduces the effective glucose concentration in contact with the enzyme while maintaining oxygen enough for the enzymatic reaction. In this case, at the steady state, the glucose concentration in the liposome core (superscript L) is lower than that in the outer solution according to the following equation:

$$\xi_g^L = \frac{\xi_g}{1 + \dfrac{V_{max} l}{k_g P_g}} \quad (3)$$

Referring to Appendix A for the detailed mathematical model, we report here only some considerations based on the assumption that ψ^2 is low and that the lipid bilayer permeability to oxygen is infinite. In this case, in order to satisfy the condition $\xi_g^L \ll \xi_o/(1 + \xi_o)$, i.e. $c_g^L \ll k_g c_o^s/(k_o + c_o^s)$, required for a linear electrode response, it is sufficient to assure that the glucose concentration in the sample is lower than $k_g c_o^s(1 + V_{max} l/k_g P_g)/(k_o + c_o^s)$ on the other hand, the desired increase in the linearity range is obtained with a reduction of the glucose reaction rate and therefore of the output current density, that is given by:

$$\eta = \frac{1}{2}\theta_h \psi^2 \frac{\xi_g^s}{1 + \dfrac{V_{max} l}{k_g P_g}} \quad (4)$$

Therefore, the lower the bilayer permeability to glucose, the wider the linearity range, but also the lower the output current density. As example, Fig. 2 reports output current trend vs. glucose concentration for enzyme-loaded liposome using $\psi^2 = 1$.

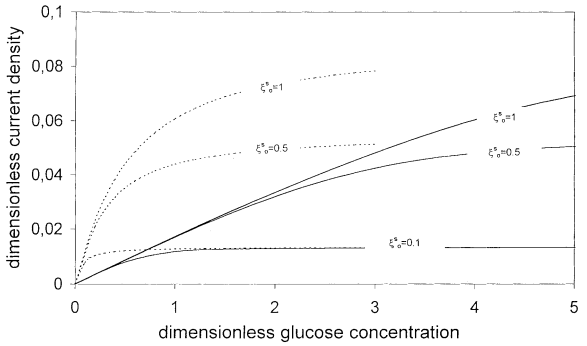

Fig. 2. Trends of output current obtained from the mathematical model for electrodes with unentrapped enzyme (dotted curves) and enzyme entrapped in liposomes (full curves), at different oxygen dimensionless concentration. Calculations have been carried out with $\theta_o = 1, \theta_h = 0.4$, $\alpha_g = \alpha_o = \alpha_h = 1$, $\psi^2 = 1$, $\beta_g = 0.1$ and $\beta_o = \infty$.

4. Experimental results and discussion

Fig. 3 reports typical amperometric responses (i.e. differences between the output current measured in the sample and the background current value at 0 glucose concentration) as a function of glucose concentration for electrode A, based on GOD or GOD entrapped in liposomes, immobilised on the Immunodyne membrane. As it is possible to note, according to the theoretical model predictions, when enzyme entrapped in liposomes is used, an increase of the linearity range and a reduction of the electrode sensitivity are obtained. In particular, with the unentrapped enzyme the output current is linear only up to

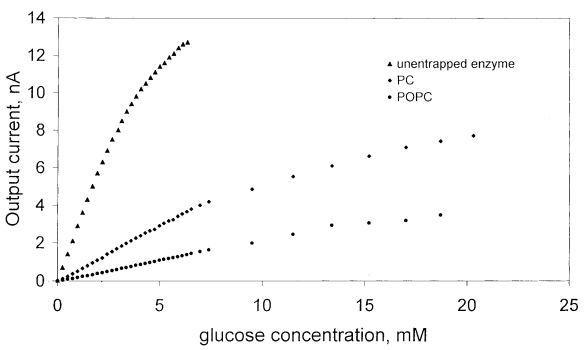

Fig. 3. Response curves as a function of glucose concentration for electrode A assembled with unentrapped enzyme and GOD entrapped in POPC and PC liposomes.

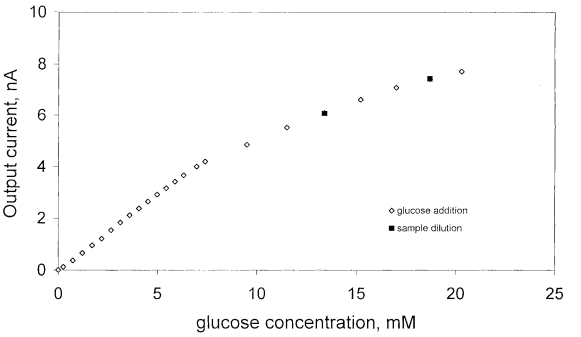

Fig. 4. Reversibility test for electrode A with PC liposomes.

about 4 mM glucose concentration; in this range the slope of the calibration curve is 2837 ± 17 nA/M. Using enzyme-loaded liposomes the output current is linear up to 10 mM for soya PC and 20 mM for synthetic POPC liposomes, whereas the slopes of the calibration curves decrease to 575 ± 2 nA/M and to 203 ± 2 nA/M, respectively. The theoretical model suggests that the entrapment in liposomes increases the upper limit of the linearity range by a factor $(1 + V_{max}l/k_gP_g)$; consequently the lower the bilayer permeability to glucose, the wider the linearity range. Therefore, the effect of lipid composition on the electrode response is related to the different bilayer permeability to glucose: POPC membranes, in fact, having POPC a lower insaturation degree with the respect to PC, show a lower permeability to glucose; therefore POPC liposomes seem to be more effective in increasing the linearity range, even if such increase is obtained with the reduction of the output current to very low values.

Experiments carried out to verify the response repeatability show that, repeating the calibration procedure with the same electrode, the same amperometric signal values within an acceptable 2.5% error limit are obtained. As far as reproducibility of measurements carried out with electrodes assembled with different portions of liposome-loaded membrane are concerned, a variability of about 15% in the slope of the linear part of the calibration curve is obtained. Finally, experiments performed by increasing the glucose concentration and, subsequently, by diluting the sample (Fig. 4) show the complete reversibility of the system.

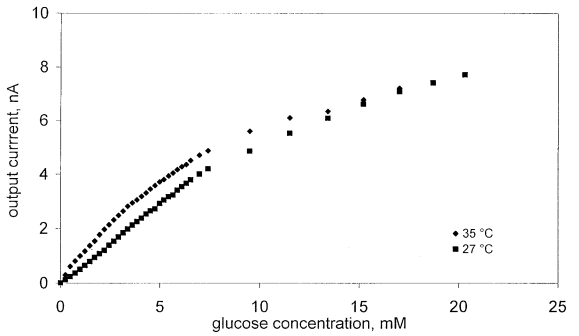

Fig. 5. Effect of temperature on the response of electrode A with PC liposomes.

Fig. 5 shows the effect of the temperature on the response of GOD/PC liposomes assembled electrodes. When temperature increases from 27 to 35 °C, the electrode sensitivity increases by a factor about 1.5. This behaviour is likely due to the increase in the glucose permeation rate through the liposome bilayer, that represent the rate limiting step. The liposomes tested do not undergo gel-to liquid crystal phase transition at these temperatures and therefore no abrupt changes in bilayer permeability are expected. Again, according to the theoretical model suggestion, the increase in the bilayer permeability results also in a reduced effectiveness of liposome to increase the linearity range.

Curves reported in Fig. 6 refer to measurements performed with unentrapped and loaded in liposome enzyme, immobilised onto SPE, in the same experimental conditions of Fig. 3. In this case, response obtained with GOD in PC liposome assembled sensor is linear up to 20 mM glucose concentration, but, as regards the sensor sensitivity, best results are achieved according with the wider active surface of these electrodes (14 502 ± 197 nA/M). A comparison of tests performed with different electrodes, following the same procedure show response curves within 25% error limit. In the same figure results obtained with the drop-on techniques, for some concentration values, are also shown. As it is possible to note higher current values are detected; this behaviour is frequently observed with SPE.

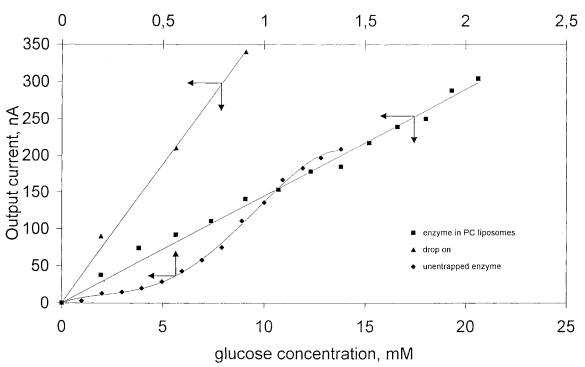

Fig. 6. Response curves as a function of glucose concentration for SPE (electrode B) assembled with unentrapped enzyme and GOD entrapped in PC liposomes. For liposome electrodes, response obtained with drop-on technique is also reported.

5. Conclusion

Experimental results confirm that electrodes assembled with GOD entrapped liposomes immobilised onto a polymeric membrane allow to obtain a wide linearity range up to clinically relevant glucose concentration; however the low permeability of the lipid bilayer decreases the electrode response to very low output current values and therefore reduces the biosensor sensitivity. The approach with SPE sensors, due to their high degree of electrochemical activity, was found to be superior and gave biosensors with higher sensitivity. Phospholipid nature influences glucose permeability and, consequently, different linearity range can be achieved using different liposome composition. Some aspects, like the sensor response stability with time and the effects of interfering substances need to be deeply investigated and work are in progress in this direction. Finally it is worth noting that the experimental results here presented agree at least qualitatively, with the behaviour predicted by the mathematical model proposed; the model itself may therefore be useful to optimise such analytical systems.

Appendix A

In a classical system, where the enzyme is immobilised in a polymeric membrane, placed on the

electrode surface, the enzymatic reaction that transform glucose (subscript g) and O_2 (subscript o) in the electro-active compound H_2O_2 (component h) is coupled with the diffusion of the various compounds in the membrane. Therefore, at the steady state, the system behaviour can be described by the following equation:

$$-D_i\frac{d^2c_i}{dX^2}+v_i v(\mathbf{c})=0 \tag{A1}$$

where D_i is the i component diffusion coefficient in the enzyme layer, v is the reaction rate, that depends on the component concentrations (vector $\mathbf{c}$) according to Eq. (1) and v_i is the stechiometric coefficient ($=1$ for glucose and oxygen, $=-1$ for H_2O_2). The following boundary conditions hold:

$$\begin{array}{llll} X=0 & c_g=c_g^s & c_o=c_o^s & c_h=0 \\ X=l & dc_g/dX=0 & dc_o/dX=0 & c_h=0 \end{array} \tag{A2}$$

where l is the thickness of the enzyme layer and the superscript s refers to the value in the enzyme layer at the interface with the sample solution ($X=0$). We assume the equilibrium conditions at the enzyme layer-solution interface, with partition coefficients α_i; furthermore the chemical reaction is considered the rate limiting step at the electrode surface ($X=l$).The output current is given by:

$$J=nFD_h\left.\frac{dc_h}{dX}\right|_{X=l} \tag{A3}$$

with $n=2$ for the hydrogen peroxide electrooxidation.

Eqs. (A1), (A2) and (A3) and the boundary conditions can be rewritten in dimensionless form:

$$\frac{d^2\xi_i}{dx}-\theta_i\psi^2 f(\xi_g,\xi_o)=0 \qquad i=g,o,h \tag{A4}$$

$$\begin{array}{llll} x=0 & \xi_g=\xi_g^s & \xi_o=\xi_o^s & \xi_h=0 \\ x=1 & d\xi_g/dx=0 & d\xi_o/dx=0 & \xi_h=0 \end{array} \tag{A5}$$

$$\eta=-\left.\frac{d\xi_h}{dx}\right|_{x=1} \tag{A6}$$

where the following dimensionless variables are defined:

$$\xi_g=\frac{c_g}{\alpha_g k_g} \qquad \xi_o=\frac{c_o}{\alpha_o k_o} \qquad \xi_h=\frac{c_h}{\alpha_h k_g} \qquad x=X/l \qquad \psi^2=\frac{V_{max}l^2}{D_g k_g}$$

$$f=\frac{\xi_g}{\xi_g\alpha_g(1+1/\xi_o\alpha_o)+1} \qquad \theta_g=1 \qquad \theta_o=\frac{D_g\alpha_g k_g}{D_o\alpha_o k_o} \qquad \theta_h=-\frac{D_g}{D_h} \qquad \eta=\frac{J}{nF\alpha_h k_g D_h}$$

The parameter ψ^2, usually referred as Thiele modulus, represents the ratio between the diffusion and reaction characteristic time.

Eq. (A4) must be solved by a numerical procedure. Here a fixed point procedure is used.

On the other hand, if the enzyme is previously entrapped in liposomes, substrate diffusion in the active layer occurs in parallel with the substrate diffusion across the liposome membrane and the enzymatic reaction in liposomes. According to this scheme, the model equation can be rewritten as:

$$-D_i\frac{d^2c_i}{dX^2}+v_i v(\mathbf{c}^L)=0 \tag{A7}$$

$$P_i(c_i-c_i^L)=v_i v(\mathbf{c}^L)\frac{R}{3} \tag{A8}$$

where superscript L refers to the concentration inside the liposome core, P is the component i permeability across the liposome membrane and R is the liposome radius. Boundary conditions Eq. (A2) and Eq. (A3) for the output current density still hold.

Eqs. (A7) and (A8) is rewritten in dimensionless form:

$$\frac{d^2\xi_i}{dx}-\theta_i\psi^2 f(\xi_g^L,\xi_o^L)=0 \tag{A9}$$

$$\beta_i(\xi_i-\xi_i^L)=v_i\, f(\xi^L,\xi_o^L) \tag{A10}$$

where $\beta=3P_i\alpha_i k_i/(RV_{max})$.

Again, the equation set must be solved numerically and a fixed point method is used.

References

[1] P. Treloar, S. Higson, M. Desai, I. Christie, S. Ghosh, M. Rosenberg, M. Subrayal, M. Jones, P. Vadgama, in: G.G. Guilbault, M. Mascini (Eds.), Uses of Immobilized Biological Compounds, Kluwer Academic Press, 1993, pp. 131–140.
[2] M.F. Rosenberg, M.N. Jones, P.M. Vadgama, Biochim. Biophys. Acta 1115 (1991) 157–165.
[3] M.A. Taylor, M.N. Jones, P.M. Vadgama, S.P.J. Higson, Biosensors Bioelectron. 10 (1995) 251–260.
[4] M.A.T. Gilmartin, R.J. Hart, C.L. Honeybourne, D. Patton, Analyst 120 (1995) 1029–1045.
[5] M.A.T. Gilmartin, R.J. Ewen, R.J. Hart, C.L. Honeybourne, Electroanalysis 7 (1995) 547–555.
[6] C.A. Galan-Vidal, J. Munoz, C. Dominguez, S. Alegret, Trends Anal. Chem. 14 (1995) 225–231.
[7] G. Bourdillon, J. Bourgeois, D. Thomas, J. Am. Chem. Soc. 102 (1980) 4231–4235.
[8] J. Raba, H. Mottola, Crit. Rev. Anal. Chem. 25 (1995) 1–42.
[9] T. Schulmeister, F. Scheller, Analytica Chimica Acta 170 (1985) 279–285.