



ALKALINE PHOSPHATASE BIOSENSOR BASED ON MODEL LIPID MEMBRANE

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ABSTRACT

Recently, solid supported thin liquid films of lipids have been recognized as prospective systems for the design of biosensors. In this study the potential of liquid film modified glassy carbon electrode for registration of phosphatase reaction is disclosed. The results suggest, that due to the different penetration of the substrate and product into the film, it is possible to detect small amounts of the enzyme in the electrolyte solution. In this regard, a brief discussion of the opportunities for implementation of sensors with electrochemical signal transduction is given.

Key words: thin liquid films, solid supported lipid layer, glassy carbon, biosensor, phosphatase, voltammetry, impedance.

INTRODUCTION

The past decades were marked by the increasing interest in the elaboration of biomembrane mimicking devices. The stability can be pointed out as one of the most tough issue to be solved for the employment of membrane model systems outside laboratory environment. In this relation, lipid layers arranged by Langmuir-Blodgett (LB) technique already proved to be reliable structures in the organization of biosensor detecting part (1-3). Another approach is based on the formation of monolayers onto solid support through the spontaneous adsorption from solution by a method known as self-assembly (4-7).

The idea for generation of solid supported thin liquid films of lipids and their use in the construction of sensors arose in pursuit of an increase of molecular mobility combined with a stability reinforced through the strong adhesion forces. Respectively, a two-dimensional (2D) design in which the detector is consisting of thin liquid layer formed onto a

solid support was proposed (8-9). Such an architecture can be realized by the aid of various amphiphilic compounds. In our case the natural lipids are namely those molecules that play the role of self-ordered elements (10). It should be noted that in addition they are able to ensure the proper micro environment which is of a great importance for the normal activity of the biomacromolecules.

Thus, the films obtained by the thinning of solid supported liquid layers become a realistic alternative of already existing techniques of lipid deposition (11-12). On the one hand, these self-organized systems offer an advantage based on the reliable compromise between their structural stability and the mobility of the constituting elements. On the other hand, as earlier results suggested, the films represent not only a selective barrier for penetration of different chemical species, but possess as well good blocking properties for heterogeneous electron transfer (13-14). These features makes them quite intriguing for the development of sensors with electrochemical signal transduction.

MATERIALS AND METHODS

1. *Electrochemical cell construction and working electrode modification.*

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The electrochemical properties of the modified glassy carbon electrode (GCE) were investigated in a two-electrode cell. It consisted of a working GCE, 0.1 mol/l solution of NaCl (Sodium Chloride, SIGMA Chemical Comp., USA), buffered with 5 mmol/l $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ – pH 9.6 (and pH 9.2 in some experiments) as electrolyte and a single junction Ag/AgCl reference electrode:

Ag / AgCl / 0.1NaCl, 0.005 $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ // LF // GC

where LF is the liquid film of lipid molecules formed onto the surface of the working GCE.

Commercially available glassy carbon electrode (GCE) with a diameter of 3mm and 0.0707cm^2 face area is used in this study as working electrode. Before each measurement the GCE surface was additionally conditioned by a fine polishing as mentioned in (10, 14). No other pretreatments of the electrode has been done.

2. Forming solution and reagents.

The liquid films deposited onto the working electrode were prepared from natural lecithin (L- α -Phosphatidylcholine, Bell Pharmacal Corp., USA and L- α -Phosphatidylcholine, from soybean, Type IV-S, SIGMA). It was dissolved in n-hexane (SIGMA Chemical Comp., USA) to give the respective concentrations.

Two types of phosphatase were used as enzyme (1. Alkaline phosphatase, lyophilized powder, $\sim 1\text{U/mg}$ protein Fluka Chemie AG, Switzerland, and 2. Alkaline Phosphatase, from bovine intestinal mucosa, lyophilized powder, ≥ 10 DEA units/mg solid, SIGMA) and α -naphthylphosphate (SIGMA Chemical Comp., USA) was used as substrate to investigate the sensitivity of the films to an enzyme reaction in the electrolyte solution.

3. Cyclic voltammetry and impedimetric techniques.

In the present work voltammetry was used as an analytical tool for examination of charge transfer between the GCE and electroactive species in the electrolyte solution. The measurements were made with commercial polarograph (OH-105, Hungary) and with potentiostat/galvanostat/FRA (VERSASTAT 3F, Princeton Applied Research, USA). The sweep rates as well as the potential range are indicated in the figure captions.

The determination of passive electrical properties of the films, hence their thickness and compactness were made by a technique based on the concept of impedance. An equivalent circuit with parallel capacitance and resistance was used to evaluate the thickness of the films. The electrochemical cell construction, the set-up and method of measurement are reported earlier (10, 13-15). The potentiostat/galvanostat/FRA (VERSASTAT 3F, Princeton Applied Research, USA) was also used to carry out the impedance measurements.

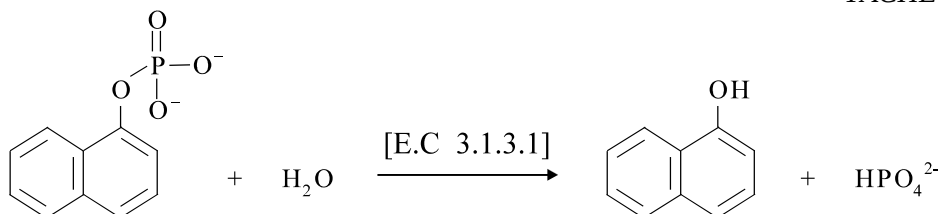
RESULTS AND DISCUSSION

The formation of the lecithin liquid films on the GCE is accomplished by a technique described in previous works (10, 13-15). Its essence is the gradual approach of the working electrode surface to the electrolyte/lipid solution phase boundary which bring the two interfaces in contact followed by a process of spontaneous film thinning. The method completely relies on the self-organization of amphiphilic molecules and enables the production of uninterrupted liquid phase of lipid. In this case the solid supported layer represents a particular branch of wetting films, namely those which is setting up a three-phase system of the type solid-liquid-liquid. In other words the system consists of an electrode (GCE), a solid supported thin liquid film of lipids and an electrolyte solution. The concepts underlying the method are related in a great extent to such areas of physical chemistry as wetting phenomena and adhesion. They are discussed in more detailed manner elsewhere (16).

The present paper is an attempt to demonstrate the capacity of coupling the films with enzyme systems for the realization of sensor elements. In this respect, the enzyme alkaline phosphatase (phosphoric monoester phosphohydrolase [EC 3.1.3.1]) has been chosen.

The phosphatases are important branch of enzymes which are known to catalyze the hydrolysis of many organophosphorus compounds. Such substances are permanently synthesized and degraded in vivo as intermediates in bioenergetics (17), as well as in chemical signals transduction on subcellular level (18).

As the enzyme retain its activity in vitro, the reaction of alkaline phosphatase is widely employed in routine clinical tests. In our case, α -naphthylphosphate is used as a substrate indicating the enzyme presence by the reaction:



Here, the product (α -naphtol) on the right side is easily electrochemically detected (19, 20), which makes the system convenient for our purposes. Moreover, due to its considerable hydrophobicity, α -naphtol is able to penetrate into the film, hence to be oxidized closer to the GCE surface, as shown bellow.

The following procedure for exploration of the film behaviour to the above reaction is used. First, a liquid film of lipids is formed onto the freshly polished GCE surface. Second, α -naphtylphosphate is added to the electrolyte (volume of 1ml) to give a final concentration of 2mM. Then two portions of the enzyme separated by an interval of 10 min. have been added to the electrolyte. Each portion contained 10 μ l enzyme with concentration of 10mg/ml (1U/mg protein). The impedance of the film is permanently measured during the whole procedure. Finally, the solution is tested for the production of α -naphtol with the aid of cyclic voltammetry.

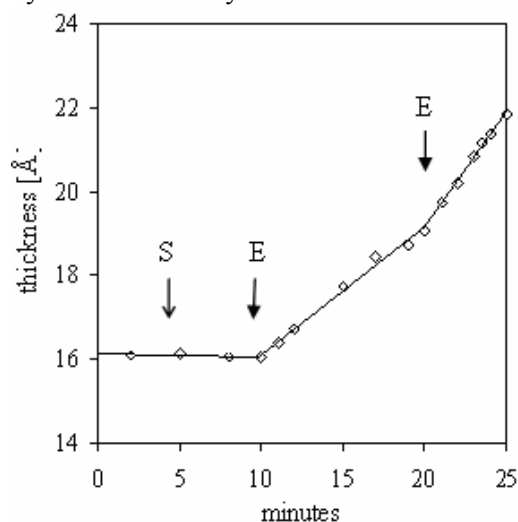


Figure 1. Changes of the film thickness upon the addition of enzyme (alkaline phosphatase, EC 3.1.3.1) in the electrolyte. The additions of the substrate S (α -naphtylphosphate) and the enzyme E are shown with arrows. The film thickness is evaluated from the impedance data at 75 Hz.

The change of the film thickness with the addition of the enzyme in the electrolyte is shown in **Fig. 1**. It is evaluated from the impedance data (at 75 Hz) according to an equivalent model with parallel capacitance and

resistance (1). Thus using the approximation of the film with a planar capacitor (21) and assuming a value for the dielectric constant $\epsilon \approx 2$ (typical for the hydrophobic core of lipid films) the apparent thickness of the film is obtained.

As seen from **Fig. 1**, a linear increase of the film thickness starts immediately after the addition of the enzyme. This shows that the product of the enzyme reaction is easily embedded into the film. Moreover, the second equivalent enzyme portion raises twice the magnitude of the slope. The evaluation with linear approximation of the changes of the film thickness gives the values of 0.3Å/min and 0.58Å/min respectively, thus indicating direct proportionality with the enzyme concentration.

Regarding the incorporation of the α -naphtol into the film, it can be said following. From the one side, α -naphtol is known to be highly soluble in nonpolar solvents, and on the other, the assumption is corroborated by the next voltammetric data.

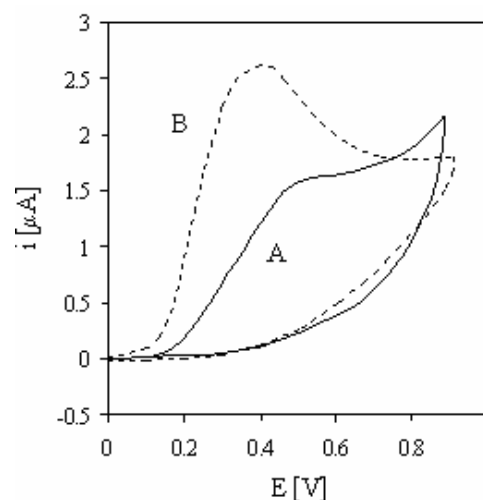


Figure 2. Cyclic voltammograms of α -naphtol generated as a product of the enzyme reaction in the electrolyte solution. (A) – modified with lipid film GCE; (B) – bare GCE. Sweep rate 2V/min.

Fig. 2 comprises two voltammograms which represent the redox reaction of α -naphtol, investigated in the potential range of 0.0V to +1.0V. The voltammogram (A) is taken at the end of the procedure discussed above when the α -naphtol (generated as a product of the

enzyme reaction) is presumably embedded into the film. The voltammogram (B) is obtained with plain unmodified with film GCE and other conditions same as (A). What the important in **Fig. 2** is, that the peaks in the oxidation currents of the two voltammograms are not shifted one to the other. This clearly suggests one and the same rate constant of heterogeneous electron transfer for the two cases thus indicating similar locations of the α -naphthol. In other words, the voltammogram (A) (**Fig. 2**) shows a diffusion limited reaction with α -naphthol capable of penetrating the film and reacting closer to the electrode surface.

The incorporation of α -naphthol into the film facilitates its registration by the impedance data, hence allowing the detection of enzyme presence. Thus it is possible to use impedimetric methods for signal transduction, an approach considered as very prospective in the area of electrochemical sensors (22-24).

To demonstrate the feasibility of implementation of the lipid films as a biosensor device for alkaline phosphatase we tested the concentration dependence of the reaction of various films. The results for three different films and respectively three different alkaline phosphatase concentrations are presented in **fig. 3**. (The alkaline phosphatase used in these experiments was from other producer and with different activity (≥ 10 DEA units/mg) as compared to the experiments presented in **figs. 1 and 2**)

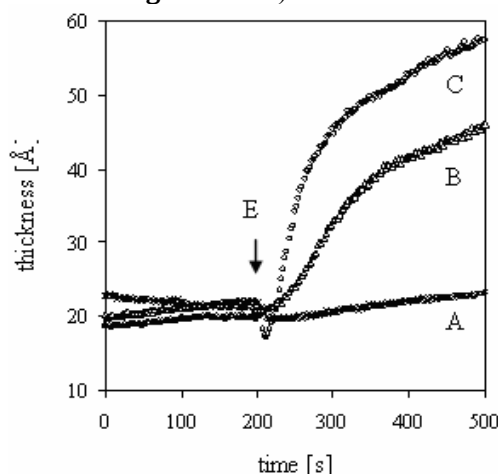


Figure 3. Changes in the thickness of three different lipid films upon addition of alkaline phosphatase at various concentrations: curve A - 4 $\mu\text{g/ml}$, curve B - 16 $\mu\text{g/ml}$ and curve C - 64 $\mu\text{g/ml}$. The substrate α -naphthylphosphate is present in the electrolyte solution before the film formation at concentration of 4mM. pH of the electrolyte solution - 9,2.

It is seen that in all three cases the thickness increases linearly initially and then reaches saturation, probably due to saturation of the film with α -naphthylphosphate. The concentration dependence of the films reaction is obtained using the initial rate of increase of the film thickness. The results are presented in **fig. 4**.

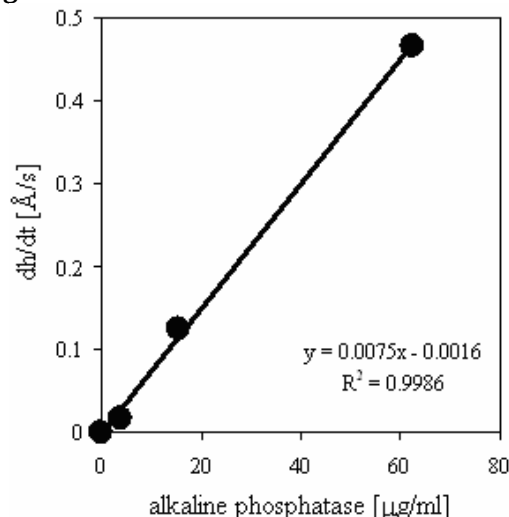


Figure 4. Dependence of the rate of the lipid film thickness change (dh/dt) on the concentration of alkaline phosphatase. Concentration of the substrate α -naphthylphosphate - 4mM. pH of the electrolyte solution - 9,2.

The experimental results in **fig. 4** are approximated with linear function. The linear dependence of the film reaction on the enzyme concentration undoubtedly demonstrates the ability to determine quantitatively the alkaline phosphatase in the tested electrolyte solution, and respectively, to use the films as a sensor element in a biosensor device.

CONCLUSIONS

As mentioned above, solid supported thin liquid films of lipids are envisioned as promising objects for biosensor construction. With their balance between stability and flexibility these self-assembled molecular structures could play a role in the design of the receptor part close to the concept of 2D arrangement. Furthermore, the formation of liquid films directly onto conducting surfaces offers possibilities for electrochemical signal transduction.

Considering the present results the abilities for combination with enzyme systems can be stressed out. As demonstrated, the interaction between a supported liquid film and the product of the enzyme reaction of alkaline phosphatase leads to readily detectable

changes in the film parameters. It should be noted, however, that the capabilities of the discussed films are not limited to this particular instance of registration of enzyme activity. Other schemes based on the protein immobilization in the films are equally possible and can be found in the literature (12, 25-27). This gives a certain motivation the films to be referred to as eventual candidates for the realization of various sensor devices. Moreover, it can be said that the development of sensors on the basis of solid supported liquid films have not only practical aspects but could add new insights to the already established concepts of the model membrane systems.

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