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Liquid photopolymerizable compositions as immobilized matrix of biosensors

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Abstract

Series of liquid photopolymerizable compositions (LPhPC) based on oligouretanemetacrylate (OUM-1000T and OUM-2000T) and oligocarbonatemetacrylate (OCM-2), monomethacrylic ether of ethylene glycol and vinylpyrrolidone (VP) were tested. It was shown that the LPhPC, which contained VP (as basic hydrophylic matrix), OCM-2 (cross-linking agent) and OUM-2000T (to increase adsorption of polymer) was the most optimal. The blend contained 3 g/100 ml of enzyme. ISFET based biosensors for analysis of glucose and urea had the following characteristics: linear response in the range of concentrations 0.1–10 mmol/l, 0.05–20 mmol/l, angle of slope of concentration curve — 30 mV/pC, 38 mV/pC, and response time of approximately 10–15, 5–10 min, correspondingly. The value of K_m for immobilized urease and β-glucose oxidase (GOD) achieved 0.85 and 3.1 mmol/l, respectively. It was established that under immobilization conditions at 20 °C the residual activity of GOD was about 35% from the initial level, the residual activity of horseradish peroxidase (HRP) and urease was 42% and 20%, respectively. In case of an immobilization of GOD at −50 °C its residual activity reached almost 50% from the initial level. It was investigated how different sources of UV radiation and different substances (including specific and non-specific substrates) influenced stability of the enzymes in the LPhPC and in the prepared membrane at storage. Dynamics of changes of enzyme activity at the process of photo immobilization was characterized, and requirements for enzyme maximal storage were selected. The proposed LPhPC may be prepared in advance since enzymes do not lose their activity during 2 months. Therefore, two processes, i.e. manufacturing of a transducer and preparation of a biological membrane on its surface, can be combined in one. In order to achieve this, approaches of modern electronics, such as for example photolithography, can be used. The developed LPhPC is homogenous, non-active to biological substances, permeable for the analyzed sample, can be prepared using a simple immobilization procedure, and has a defined hydrophobic–hydrophilic balance and sufficient level of adhesion to transducer surfaces. These all cover the requirements to modern biosensors.
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1. Introduction

Early [1–4] we developed prototypes of the enzyme- and immune biosensors based on the ISFETs and the electrolyte insulator semiconductor (EIS) structures. Both types of biosensors are perspective for use in different fields, in particular, medicine, biotechnology and environmental monitoring. However, in order to start their wide manufacturing it is necessary to optimize the procedure of immobilization of a biological material on the transducer surface. Generally it is considered to be the main problem of biosensors and in order to solve it different approaches, e.g., physical, chemical and hybrid physical–chemical methods were proposed [5–7]. All these methods are aimed at a better fulfillment of the main requirements, related to a maximum level of the residual activity of biological molecules, a better exposition of active centers towards a solution with analytes, simplification of the immobilization procedure, possibilities to combine the preparation of a biological membrane with the electronic cycle of transducer manufacturing and preservation of a high level response during storage of the biosensor, etc.

The use of the liquid polymerizable compositions (LPC) on the basis of monomer-oligomeric substances to create a biological

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membrane may be considered a perspective approach allowing the satisfaction of the above-mentioned demands. These compositions give a possibility to form sensitive membranes with adjustable physical–chemical and mechanical abilities without strong temperature and chemical destructive effects on biological molecules. Monomeric and oligomeric acrylate compounds (acrylic, metacrylic acids their ethers and derivatives), urethane oligomers and vinyl copolymers (sterol, vinyl acetate, vinylidenchloride, vinylpyrrolidone and others) are those, which belong to the category of the most widespread LPCs. Varying chemical origin and concentration of some of the components it is possible to regulate different parameters of biological membranes obtained on the basis of these components.

Because of the use of the LPC in biosensors, it is expected that such biosensors would be characterized by non-activity to biological substances, permeability to the analytes and a sufficient level of adhesion, defined by hydrophobic–hydrophilic balance, to a transducer surface. Therefore, the liquid photopolymerizable composition (LPhPC) attracts a special interest, even though its wide application is restricted by the requirements summarized above. As a rule, the influence of supportive substances on biological materials is not specially studied in biosensors. Usually an excess of a biological material is taken and for the estimation of its state non-direct approaches are used, namely, the determination of biosensor response, the rate of product formation and others. At the same time, any changes in the structure of biological molecules, which can occur during the creation of biochips or during their preservation, would affect significantly the intensity of response and lifetime of such a biosensor. Moreover, when a multi-layer immobilization of a biological material is used, the inner layers may work with a lower productivity compared to the productivity of the external layers. Such a difference in productivity may be caused by restrictions for diffusion. That is why the main objective of our work, the main results of which are described in this paper, was to find a content of the LPhPC, which would be distinguished by a number of characteristics satisfying the current requirements set for biosensors: simplicity of immobilization procedure and homogeneity of formed membrane. To optimize the conditions of the enzyme inclusion in the LPhPC the absolute level of residual activity of the immobilized molecules was determined and principal factors affecting this level were characterized.

2. Materials and methods

In our experiments we used urease from soybean with activity of 200 u/mg (Sigma, USA), glucose oxidase (GOD) from *Penicillium vitale* with activity of 160 u/mg (Kamenskoe distillery, Ukraine) and horseradish peroxidase (HRP) of type VI with activity of 275 u/mg (Sigma, USA).

N-vinylpyrrolidone (VP) was obtained from "Aldrich" (Germany). 2-Hydroxy-2-methyl-1-phenylpropan-1-one (Darocure 1173, λ_{max}=310–350 nm) from "Ciba-Geigy", Switzerland, served as a photo initiator (PhI). Monomethacrylate ether ethylene glycol (MEG) was produced by "BASF" (Germany) and oligocarbonatediethylenglycolmetacrylate (OKM-2) by AOOT "Korund" (Russia). Oligouretanemetacrylate (OUM-1000T or OUM-2000T) was synthesized according to [8].

The ISFETs were manufactured in the Institute of Biocybernetics and Biomedical Engineering of PAN (Poland). Each chip contained two ISFETs, which were characterized by 45–48 mV/pH. Construction of the ISFETs, device for registration of their response and the main algorithm of measurement were described early [4]. The gate surface of the ISFETs was preliminary cleaned by consecutive washing: sulphuric acid, water and ethanol. On the top of this surface the mixture of the appropriate enzyme and the LPhPC (about 1–5 μl) was dropped. Polymerization of this mixture was carried out using the ultraviolet (UV) radiation in vacuum conditions (0.1–0.2 mm of mercury). As a source of the UV the following lamps were used: LUF-80–04 (λ_{max}=300–400 nm, intensity of light on the irradiated surface — about 2.6 W/m^2) and DRT-120 (λ_{max}=320–400 nm, intensity of luminous flux about 12.5 W/m^2).

The homogeneity of the mixture and obtained polymer was determined visually: absence of visible disseminations using a microscope was studied at a maximal level and was marked as (++). Adhesion abilities of the formed polymer were determined by calculating the time during which no peeling of the membrane on the transducer surface was observed at the immersion of chip into a buffer solution. Extreme observations, such as immediate peeling of membrane or its intact state for 2 months were marked by (−−) and (++), correspondingly. When the residual enzyme activity was determined, the LPhPC was prepared using a two-component mixture containing VP and PhI at 98 g/100 g and 2 g/100 g of concentration, respectively. Then, 50 μl of this mixture was mixed with 20 μl of the enzyme solution, vibrated and water was removed at the vacuum conditions (0.1–0.2 mm of mercury). The concentrations of urease, GOD and HRP in the solutions were 0.1, 0.1 and 0.02 mg per 1 ml, respectively. The time of UV irradiation provided by LUF-80–04 and DRT lamps was 11 and 4 min, respectively. Intensity of luminous flux was measured by the automotive dosimeter (DAU-81). Part of the obtained membrane was dissolved in 2 ml of 10 mM phosphate buffer with pH

Table 1
Some characteristics of the LPhPC based on VP[a]

VP/ mas.%	MEG/ mas.%	OKM-2/ mas.%	OUM-1000T/ mas.%	Homogeneity		Adhesion of membrane to ISFET surface	Response on 10 mM GOD[b]
				Mixture with GOD	Membranes		
88	10			−−	−−		−
93	5			−	−		−
88	5	5		−	−	−	12
88		10		++	++	+−	42
78		20		++	+	−	33
78		10	10	+	+	++	46
68		10	20	−	−	++	25
78		5	15	−	+	++	40
78			20	−−	−−	++	20
78		10	10[c]	+	+	++	57

[a] Quantity of PhI in all LPhPC was 2 mas.%.
[b] In 1 mM sodium phosphate buffer, pH 7.0.
[c] Instead of OUM-1000T, OUM-2000T was used.

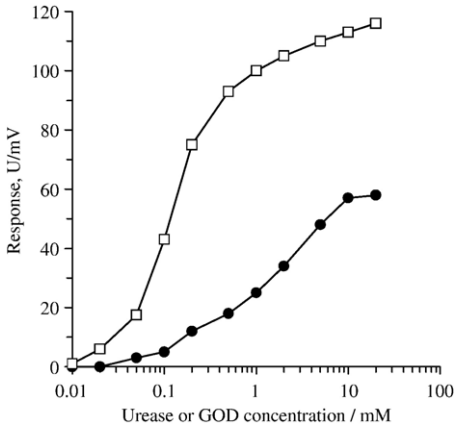

Fig. 1. Responses of biosensors with the immobilized GOD (●) and urease (□). Measurements were fulfilled in 1 mM of sodium phosphate buffer, pH 7.0.

of 5.5, 7.0 and 6.0 in the case of the determination of activity of GOD, urease and HRP, respectively.

In order to obtain calibration curves, the VP, polyVP (PVP) or intermediate products of these substances (depending on duration of irradiation or method of analysis) were added to the analyzed samples. Other details of experiments are given in the text below.

The obtained results were analyzed statistically using the *t*-criteria.

3. Results and discussion

According to preliminary investigations, the VP was taken as main component of the LPhPC, because it had the most appropriate hydrophilic–hydrophobic balance. The optimal contents of the enzymes and PhI were 3 and 2 g per 100 g of LPhPC, respectively. Primarily MEG was used as cross-linking polymer.

The results of choosing an optimal variant of the LPhPC with respect to homogeneity of the obtained polymer, its adhesion to the transducer surface and the biosensor response are summarized in Table 1.

The response curves of the GOD and urease biosensors at the presence of substrates in different concentrations are summarized in Fig. 1. The biosensors had the following characteristics: the linear region of responses was 0.1–10, 0.05–20 mmol/l, slope of curve — 30, 38 mV/pC and response time — 10–15, 5–10 min for glucose and urea, respectively (Table 2). The K_m values for enzymes were calculated using the method of double reverse figures [9]. These values for GOD and urease were 3.1 and 0.85 mmol/l, respectively.

Table 2
Some characteristics of the immobilized enzymes and appropriate biosensors

Enzyme	Slope of curve, mV/pC	Linear region of response/ mM	K_m of native enzymes/mM	K_m of immobilized enzymes/mM	Response time/min
GOD	30	0.1–10	6.66	3.1	10–15
Urease	38	0.05–20	2.9	0.85	5–10

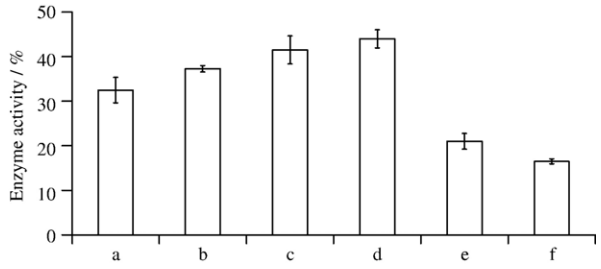

Fig. 2. Residual activity of GOD (a, b), HRP (c, d) and urease (e, f) in the LPhPC. Source of irradiation: a, c, e — LUV and b, d, f — DRT lamp.

The LPhPC, which contained VP, OKM-2 and PhI in the ratio 78:10:2 g/100 g, showed to be the most optimal.

Often mixing of the LPhPC with a buffer solution aggravates homogeneity and mechanical abilities of the obtained membrane due to presence of salt ions. We tried to exchange a buffer solution with distilled water; however, we found that such an exchange did not affect the residual activity of enzymes in the LPhPC. The presence of VP in the solution did not affect the enzymatic activity either.

Further it was demonstrated that the activities of GOD and HRP did not differ statistically when different LUV-80–04 and DRT-120 lamps were used. Urease, however, behaved differently. The DRT lamp caused the strongest effect. The level of activities of the native and immobilized enzymes before and after UV irradiation from different sources is given in Fig. 2.

To determine effect of the UV radiation with different wavelengths on the enzyme activity, short waves (up to $\lambda = 300$ nm) were removed using a glass filter (thickness of 3 mm). Irradiation of the mixture by the DRT lamp leads to an increase of the residual activity of urease. Such a difference in the effect of the LUV and DRT lamps may be caused by the presence of short waves (less 300 nm) in the spectrum of the DRT lamp that has a negative effect on the urease.

To choose an optimal duration of the LPhPC irradiation by the LUV lamp, intervals 220, 330, 440, 660 and 990 s were examined. Maximal changes of the urease activity were observed after 300–420 s of the LPhPC irradiation. The intensity of absorbance at 1700 cm^{-1}, which corresponds to double carbon–carbon bonds in the VP, was determined with the help of an infrared spectrophotometer (SP-300S Philips). A

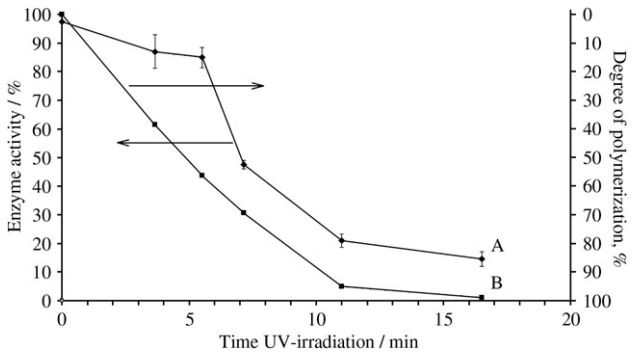

Fig. 3. Dynamics of the urease activity changes and the level of the LPhPC polymerization in dependence on duration of UV irradiation by the LUV lamp.

decrease of enzymatic activity was found to correlate with the level of polymerization of the LPhPC (Fig. 3).

It is well known that the enzyme activity is preserved well at low temperatures. That is why we tried to decrease the temperature of the LPhPC at the immobilization of urease up to −8 °C. However, the immobilization had to be done in a solid phase, because the LPhPC is known to acquire its solid phase state at +13 °C. Unfortunately decrease of the LPhPC temperature did not have any effect on the level of the residual urease activity. Nevertheless sharp decrease of the LPhPC temperature leads to the preservation of the GOD activity. So, at the polymerization temperature of about −50 °C the GOD activity was 50% higher than when temperature was +20 °C (Fig. 4).

We also examined a possibility to protect enzymes during the immobilization process by the use of non-catalyzed substrates. Unfortunately a compound similar to the urease substrate was present in all solutions of the LPhPC. In order to avoid its presence in the LPhPC a special algorithm of analysis was developed. Firstly, the LPhPC containing OUM-2000T, VP and PhI in the ratio of 10:88:2 g/100 g was prepared. Then, this composition was solidified after introduction of the enzyme and its non-catalyzed substrate. This resulted in a strong three-dimensional and good elastic polymer. From this composition films with thickness of 0.1–0.15 mm were prepared, which contained thiourea and in which thiourea was absent. Thiourea was removed at the washing of films during 2 days. The enzymatic activity was calculated per square unit of the surface. It was shown (Fig. 5) that thiourea had a maximum protective effect (11.3%, $p<0.05$) at its concentration of 0.5 g/100 g of the LPhPC.

At the preservation (4 °C in freezer) of the prepared membrane the urease activity decreased with 15% ($p<0.05$) during 2 months. After 6 months the decrease was 47% ($p<0.005$). At the same time, at the preservation of urease in the LPhPC during 2 months its activity did not change. The decrease of the urease activity was observed only after 6 months; it was 30% from the initial level ($p<0.01$). In case of the preservation of the membrane with GOD during 6 months the enzyme activity decreases on 23% only ($p<0.005$).

In conclusion, optimal conditions were proposed for the preparation of the LPhPC with an increased homogeneity and adhesion abilities and for the preservation of the enzyme activity in the obtained membrane. The proposed composition may be prepared in advance since enzymes did not lose or lost only a bit of their activity during 2 months. In addition, manufacturing of transducers and simultaneous preparation of a biological membrane on their surface may be combined in one process using traditional approaches of modern electronics, e.g., photolithography.

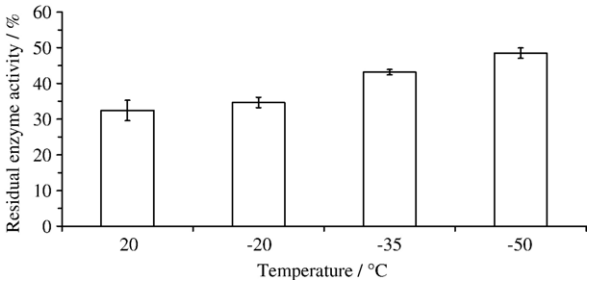

Fig. 4. Residual GOD activity in dependence on the LPhPC temperature.

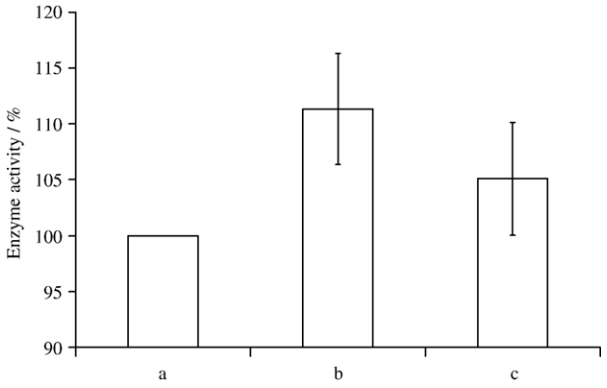

Fig. 5. Level of urease activity (% from initial one) in dependence on the concentration of thiourea in the LPhPC, where: a — 0, b — 0.5, and c — 1.0 g/100 ml.

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