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Photopolymerizable Materials in Biosensorics

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1. Introduction

The development of the effective methods for the biological material immobilization is the main problem of biosensorics. This process may be classified as including biological selective components into isolated phase which is separated from free solution but can exchange with its by molecules of substrate, effectors, inhibitors and others (Triven, 1983). The most often biological material is covalently bound with some insoluble polymer, linked together or with some inert protein. In all these cases it is obtained the non-soluble but active complex. It is realization of chemical approaches which have unfortunately a number of disadvantages and main among them is the lost of activity of biological materials (and often very much). Another set of methods is associated with the physical sorption of biological material on the transducer surface at the use of electrostatic or non-covalent mechanisms of binding. In this case, as a rule, the loss of biological material activity does not occur but for the providing a reliable binding there is necessary to complicate immobilization procedure. Application of poly-electrolytes as intermediate layer is the most productive way. From other side, biological material may be not directly bound to the some surface and can be kept inside of a special polymer or double phospholipids (liposomes).
The choice of a particular method of immobilization is a very important moment in the development of biosensors and it must be based on taken into account of the following points: 1) what kind of chemical or physical-chemical reactions will be occurred on the surface; 2) molecules should kept the stability at the process of immobilization and during chip working; 3) chemicals for cross linking should interact with groups of biomolecules which are remote from their active centers; 4) if demands of point 3 can not be fulfilled the bifunctional reagents which are used for the linking should be as large as possible to penetrate to the active centers of biomolecules (for example, activated cellulose is more suitable than glutaraldehyde); 5) active sites must be protected, in particular, by substrates or glutathione, cysteine, papain or others reagents for blocking sulfhydryl groups with their reactivation in advance; 6) the procedure of washing of not-linked biomaterial should not effect negatively for immobilized one, especially, if there are subunit forms to prevent their dissociation; 7) what kind of physical and mechanical abilities may form immobilized material: thin layer, thick film, an amorphous structure, etc. If all these points are correspond to needed conditions the chosen method is well for the creation of biosensor.

2. The application of polymers as immobilization matrix in biosensors

One among of simple and reliable approach for integration of the biomaterial (enzymes, antibodies, antigens, cells) in biosensors is based on the use of polymers (Rehman et al.,

1999; Turner, 1989). The first investigations in this direction were fulfilled shortly after the discovery of this type of instrumental analytical devices by Clark (Turner, 1989). In most cases it was the polymers obtained by chemical way from acrylic acid and used for including enzymes and cells (Freemen, 1986; Starodub et al., 1990; 1995; 1998; 1999; 2001). These investigations shown as perspective application synthetic polymers in biosensor technology and opened all problems in this respect. Up to now a lot of work ware curried out in this aspect.

Recently, miniaturization of the transducers and the application of number of different biological components in the same biosensors are occurred. Unfortunately, the traditional approaches can not fulfill all practice demands in this aspect. Only photochemical formed and cross linked polymers may meet the requirements of technology of biosensors. The application of such approach has the next advantages at the currying out of the immobilization: 1) absence of destructive factors; 2) possibility to work at the room or more low temperatures; 3) accurate given initiation and termination of process; 4) formation biopolymer in precisely defined area, usually in a very small; 5) combination in single technological process of photolithography production of semiconductors. All this could affect significantly on the production costs and expanding its application (Grishchenko et al., 1985; Masljuk & Chranovsky, 1989; Starodub, 1989, 1990; Kuriyamma & Kimura, 1991).

2.1 General characteristics of the formation of photopolymers

For the activation of process of the photochemical polymerization it is used ultraviolet (UV) and not so often fast electrons, roentgen, gamma and plasma radiation. Certainly, these factors damage biological materials so try to use less stringent radiation, for example, UV with wavelength within 300-400 nm. The photochemical transformation of unsaturated compound in the polymer is activated with the help of photo initiators (PhI), which can absorb photons of UV. At that energy of the activation of polymerization process decreases (up to 17-34 kJ/mol) in comparison with the photo initiated solidification. Absorption of light by PhI transforms of its in electronically excited state which causes destruction of molecule with the formation of free radicals initiated polymerization. PhI is usually thermally stable (Masljuk & Chranovsky, 1989). Among of photochemical reactions used for obtaining polymers having practical importance there is necessary to pay attention on the photo destruction (photochemical cross-linking) and photo polymerization. Two types of photocomposition deserve attention. First type of structuring consists in cross-linking of preliminary obtained linear polymer due to taken part of side reaction groups in the presence of PhI (or without it) at the UV irradiation. Second type of photo structuring is photochemical formation of polymers with the participation of bifunctional light sensitive substances. In this case photo cross-linking of linear polymers is accomplished through a special photo sensitive reagent. There is necessary to underline that the obtaining polymers through photochemical initiation is used in industry more often than through photo cross-linking. Oligomers, which are able to polymerization and contended inter chain and terminal double bonds as well as light-sensitive monomer-oligomer compositions based on them, at the exposition to UV radiation solidify, forming a polymer material. The preferential use of these classes of substances at the obtaining of photo polymerisable compositions is connected with the great potential to regular properties of oligomers by changing the nature and structure of the starting compounds for synthesis or as a result of copolymerization with many vinyl monomers, which ultimately determines the possibility of creating complex polymeric materials with different properties. The widespread used

compositions of photo polymerizable compositions in biosensors are based on the derivatives of acrylic acid and polyurethanes as well as in the case of photo-cross linkable polymers polyvinyl alcohol and polyvinylpyrrolidone. They are the most dispersed in application. Below we will concentrate our attention on the features of the procedures and preparation photo polymerisable and photo-cross linkable polymers.

2.2 Photo polymerization in biosensorics

We will discuss about two different approaches in the formation of photopolymers, namely, with application of acrylic and urethane derivatives.

2.2.1 Acrylic derivatives

These derivatives as photopolymers were used often for the creation of biosensor elements (Arica & Hasirci, 1987; Kumakura & Kaetsu, 1989; Doretti & Ferrara. 1993; Doretti et al., 1994; Jimenez et al., 1995; Macca et al., 1995; Moser et al., 1995; Gooding & Hall, 1996; Lesho & Sheppard, 1996; Ambrose & Meyerhoff, 1997; Wróblewski et al., 1997; Doretti et al., 1998; Hall et al., 1999; Kolytcheva et al., 1999; Mohy et al., 1999; Rehman et al., 1999). 2-(hydroxyethyl)methacrylate (HEMA) is used the mostet often. The enzyme immobilization in polymeric matrix on the basis of HEMA has some advantages since it has appropriate mechanical abilities and optimal pore size needed for the retention of enzyme molecules, transportation of substrates and products of reaction.

It was reported (Arica & Hasirci, 1987) about β-glucose oxidase (GOD) immobilization in polymeric gel contended HEMA and N,N′-methylenebisacrylamide (gross-linking agent). Azobis-izonitril and ammonia-persulfat served as PhI. Mixture of monomers, PhI and enzyme dissolved in phosphate buffer (0.1 M, pH 7.0) were illuminated by UV lamp (12 W) at the temperature of 25 ˚C in nitrogen. The immobilized enzyme had maximal activity at pH 7.0 and 35 ˚C (in opposite 5,5 and 30 ˚C in free state) with some higher value of K_M (13.33 mM comparatively 6.66 mM) and decreasing maximal reaction rate in 1.6 time. The residual activity of the immobilized enzyme after 60 days of preservation was ~90% of initial level.

The main reason for a significant change in the pH optimum of immobilized GOD (7.0) is, apparently, a local pH decreasing in the membrane during enzyme functioning. We should also mention about some diffusion limitations in the kinetics of reactions catalyzed by immobilized enzymes, leading to an increase in the apparent K_M.

Other researchers (Kumakura & Kaetsu, 1989) used hydroxyethyl, HEMA and tetra-ethylene glycol diacrylate as monomers for immobilization of cellulase. Polymerization was induced by γ-rays of cobalt-60 (exposure time 1 hour, 1 Mrad dose, the temperature of 24˚ C or -78˚ C). The polymerization rate increased with the addition of water to HEMA. It was studied the dependence of the enzyme activity on the ratio of water-HEMA and the thickness of the obtained membrane (0.1 - 1 mm). When HEMA content was 20% the cellulase activity increased with the membrane thickness. At the HEMA content of 60% the observed maximum was at 0.6 mm and with pure HEMA - gradual decline of enzyme activity with increasing membrane thickness. When HEMA content was 80% and membrane thickness in 0.1 mm enzyme activity was the same as in case of HEMA content of 20% and 1 mm thick membranes. The reason is that with the raise of water content in the polymer its hydrophilic properties are increased. It promotes to the establishment of native conformation of the enzyme and, moreover, polymer with a water content of 60-90% has a high porosity (pore diameter is 2-5 μm) and enzyme molecules are rapid washed from the membrane. When the water content is about 20-30% the pore diameter reaches 0.2-0.5 mm and although it is larger

than the size of the enzyme molecule the polymer keeps an active immobilized molecules. At the use of pure HEMA the obtained polymer has not a porous structure that prevents the penetration of the substrate to the enzyme. Membranes obtained from other monomers (hydroxyethyl acrylate and tetraethylene glycol diacrylate) showed the worst enzyme activity.

A somewhat different method was proposed (Doretti et al., 1998) based on the use of a mixture of HEMA (83%), glycidyl methacrylate (13%) and 4% of trimethyl-propan-trimethacrylate (cross linking agent). This mixture was polymerized by irradiation of γ-rays from cobalt-60 at -78 ˚C. To the resulting polymer, the enzyme was linked by interaction of amino groups with the methacrylate copolymer (polymer solution contacted with the enzyme). Amperometric transducer (platinum wire) was coated with this polymer to which then butyryl- or acetyl cholinesterase, or cholineoxidase, or peroxidase was linked. It was obtained a linear response to acetylcholine chloride - $5 \cdot 10^{-6}$ – $1.4 \cdot 10^{-4}$ M and for iodide butirilcholine - $2 \cdot 10^{-6}$ - 10^{-4} M, with an optimum at pH 9.5 and 8.0, respectively.

Nylon membrane (with pores of 0.2, 1.2, 3.0 μm) was treated with diethylene glycol dimethacrylate (acetone solution) under the influence of γ-rays (Cesium-137 source). They were then exposed alternately in solutions of glutaraldehyde and β-galactosidase. The best results were achieved using a 15% solution of diethylene glycol dimethacrylate, 2.5% of glutaraldehyde and the enzyme concentration of 10 mg/ml. The optimal response biosensor was achieved at a temperature of 50-60 ˚C and pH 4-6. It is expected the use of membranes prepared by the above mentioned method to create thermal bioreactor designed for the determination of glucose in the milk (Rehman et al, 1999). There is evidence about linking oligonucleotides to optrodes with help of acrylamide. For this purpose, the surface was treated by 3-methacryl-oxy-propyl-trimethoxy-silane under the ultraviolet light, as well as acrylamide and bisacrylamide in a ratio of 17:1 by weight. Oligonucleotides contended 5'-terminal acrylamide group covalently were linked to above mentioned surfacee. The density of immobilized oligonucleotides was 190 - 200 femtamol/mm^2.

For the immobilization of penicillinase or penicillin amidase it was proposed a approach (Macca et al., 1995) using a mixture of HEMA, N, N'-methylenebisacrylamide and enzyme solution in phosphate buffer. This mixture is sprayed into cooled n-hexane (-78 ˚C) and irradiated with γ-rays of cobalt-60. As a result of this procedure the spherical polymer beads with a diameter of about 0.5 mm were obtained and used in a flow pH-sensitive bioreactor. It was found that the sensitivity of the biosensor with such membranes was $6.3 \cdot 10^{-3}$ M of benzyl penicillin sodium for both enzymes and a change of its response level reached about 91 mV/decade of concentration for penicillinase and 66 mV/decade of penicillin amidase.

At the creation of an amperometric biosensor for the measurement of glucose the enzyme was mixed in buffer (pH 7.0) with acrylamide, N, N'-methilene diacrylamide, 2,2-dimethoxy-2-phenylacetophenone (PhI) and glycerin. The polymerization was initiated by UV. The biosensor had sensitivity from 45 to 67 nA/mM, the linear response region was located in zone $4 \cdot 10^{-3}$ - 1 mM. About 95% of the maximum response biosensor with a membrane thickness of 10-15 μm was realized within 20-30 sec. It was stable for 3 weeks (Jimenez et al., 1995).

Another amperometric biosensor for glucose (Doretti&Ferrara, 1993) was obtained as follows. HEMA and trimethylol-propan-triacrilate (cross-linking agent) in a ratio of 96:4 was mixed with a solution GOD in 0.1 M phosphate buffer (at a ratio of 2:1). Enzyme concentration in the mixture was 4 mg per g of mixture which was polymerized at -78 ˚C and with the application of cobalt-60 γ-rays. The optimal level of the response of the

obtained biosensor was at pH 6.0 and 40 ˚C (in compared with pH 5.5 and 45 ˚C for the free enzyme). Biosensor response time was about 2 min and its linear region was within $5 \cdot 10^{-5}$ – $1.2 \cdot 10^{-3}$ M. The loss of enzyme activity for the month was 25%. It should note that the polymers considered types were used for the creation of the chemical sensors too. Thus, at the development of potentiometric and optical devices for the measurements of polyanions (Ambrose & Meyerhoff, 1997) photosensitive thin films based on decylmetakrilate (DMA) were applied. In accordance with the existing theory, the regulation of the potentiometric response to the polyanion, in particular, to heparin, increases with decreasing amounts of plasticizer and tridodecyl methyl ammonium chloride (exchanger) in the film. By varying the content of cross linker in the DMA film provides an additional mechanism for the regulation of expression of its physical structure and appearance of potentiometric answer on polyanion. Films with low hexanediol-di-methacrylate as a cross linking agent are provided the detection of heparin at 0.04 mM with a low coefficient of diffusion within the polymer due to interactions between adjacent residues of decile groups. Increase of the cross-linking agent violated these interactions and increased the diffusion properties of the polymer. When applying such films to the glass surface of the optical transducer the sensitivity analysis of heparin in non-dissolved human plasma was achieved at 0.5-5.0 units/ml.

It was studied the effects of the immobilization matrix (polyacrylate nature) on the properties of ion-sensitive field effect transistor (IsFET) based sensor at the determination of K+, NO3-, Ca2+ ions (Kolytcheva et al., 1999). The membranes with two matrixes were used. The so-called PA-matrix was prepared on bisphenol-A-diglycidyl-methyl-methacrylate (BIS-GMA) and hexandiol-acrylate (HDDA). PA-matrix II consisted of n-(ethylene oxide)-dimethacrylate ((EO)nDMA). Photo initiators were phenantrenquinon and lutsirin, respectively. To determine the K+, NO3- three types of (EO) nDMA were as most suitable. They contended the three ethylenoxide groups in the monomer ((EO)3DMA). These polymers had a structure polycycles. Homologous derivatives (with the number of ethylenoxide groups) were ineligible due to the short period of existence (15 and 10 min, respectively) as the result of plasticizer leaching. Among of four plasticizers - dibutyl sebacynate (DBS), o-nitrophenyl-n-octyl ether (NPOE), di-octyladipate (DOA) and di-octyl phthalate (DOP) for a polymer matrix based on (EO)3DMA the better chemical sensor characteristics were obtained for DOP. It was found the optimal content of DOP (by weight) for K+ and NO3- membranes (45% and 32%) respectively). For ionophore it was 4% and 3% in the case of valinomycin and tributyl-oktadecyl-phosphonia, respectively. Response of these sensors reached 54.5 and 56.2 mV/decade and the defined minimum was $0.43 \cdot 10^{-4}$ and $0.19 \cdot 10^{-4}$ M for the duration of operation of 3 and 1 month, respectively. Sensors based on polymer PA-matrix II showed better results compared with those which were prepared on the basis of PA-matrix I, and polyvinyl chloride. For Ca2+-sensor it was found the best content (by weight) of plasticizer - 32% for PA-matrix I and 54% for PA-matrix II and ionophore - 3%. The mechanical properties of membranes, their performance and selectivity were the best on the basis of PA-matrix I, while the reproducibility and stability of membranes was improved by the use of PA-matrix II. The best answer had sensors based on PA-matrix I with the inclusion of Ca-ionophores ETH 1001 (Fluka), N', N', N',-N'-3-tetracyclohexyl-3-oxapentandiamide (for 7 studied ionophores) and plasticizer ETH 469 and ETH 2112 (Fluka). When stored sensors contained ETH 469 and ETH 2112 in a solution of 0.125 M KCl and 0.1 M NaCl they remained stable for 7 and 10 days, respectively. Nevertheless, the best results were obtained at the use of PA-matrix I, ETH 469 and

N',N',N',-N'-tetra cyclohexyl-3-oxy-pentane-diamid (in the proportions given above). Linear response of sensors with these membranes was in range of 10-5 – 10-1 M, the slope - 24.5 mV/decade, the response time - 40-100 sec. In the case of PA-matrix II, it was found that the ionophore ETH 1001 and plasticizer DOS were better.

2.2.2 Urethane derivatives

Polyurethane derivatives proved to be very promising in this direction (Munoz et al., 1997; Puig-Lleixaet al., 1999a; 1999b; 1999c). On their basis the sensor for the determination of monochlor acetate was developed (Puig-Lleixaet al., 1999a). As an olygomer it was used aliphatic urethane diacrylate and hexandiol-diacrylate served as gross-linking agent (their ratio was 81:17). 2,2'-dimethoxyphenyl-acetophenone was as PhI. It was chosen the most suitable ion-selective ionophores (tetradecyl ammonium bromide and tetraoctyl ammonium bromide). The plasticizers were selected from bis-(2-ethylhexyl) sebacynate (DOS), dibutyl sebacynate (DBS), di-5-noniladipata (DNA), bis-(2-ethylhexyl) phthalate (DOP) and trioctyl-phosphate (TOP). Ion-selective membrane was formed by applying 100 ml of the membrane cocktail on the surface of the transducer covered with a mixture of epoxy and graphite and irradiated by UV light (365 nm). At the selecting the components of membranes and their relationship it was preferred ammonium bromide-tetradecyl as ionophore. It was found that the best results are obtained by using DOS. Moreover, it was shown that an increase (60%) of plasticizer leads to a significant expansion of the linear response and sensitivity of the biosensor. Optimal content ionophore was at 1%, as it increases to 5-10%, though increases the sensitivity to analyte, but leads to a significant drop of this index during further operation. It was studied the interfering effects of various ions (tris, chloride, nitrate, sulfate, phosphate) in response of the sensor when above mentioned plasticizers were used. The widest range of linear response was obtained when DOS, DNA and TOP were used. Membranes contained DOS and TOP showed the least interference and membranes based on DOS showed a lower limit of the determined substance. The choice was made in favor of DOS using as the plasticizer. Sensitivity biosensor based on the selected components was 54.6±2,3 mV/decade of monochlor acetate, the region of linear response was in the range of $2.1 \cdot 10^{-5}$ – 0.1 M. The response was stable under the pH change from 10 to 4. Response time was less than 18 sec when the 95% of its value was realized (at the concentrations of analyte about 10^{-3} -10^{-2} M). Stability of response was maintained for 90 days.

This same group of authors (Puig-Lleixaet al., 1999c) suggested that the pH-sensitive sensor based on a prepared membrane using a polymer composition, similar to the previous one, but with the inclusion of thri-dodecylamine as ionophore to hydrogen cation and tetrakis-(p-chlorophenyl) borate (KTpClPB) as a cation-exchange site for potassium. As suitable buffer solution it was 0.01 M tris-HCl. Among of four plasticizers (DOS, DNA, dibutyl phthalate – DBP, DOP) the first two were as most suitable for membranes with which the upper limit of sensitivity was more advanced. However, preference is given to DOS because of the greater its resistance to ions of potassium and sodium. Selected photo polymerizable membrane consisted from 42% urethane diacrylate, 55% DOS, 1,5% ionophore, 0,5% KTpClPB and 1% PhI. Sensitivity of the sensor reached 55,4±1,5 mV/pH. When storing the membranes during 9 months their mechanical properties are maintained and reducing the sensor response was only 4%. Notes, that at the operation of the sensor the decrease of its response value was faster than at storage. With the use of the above mentioned acrylurethane oligomers, cross linking agents (tri-propylen-glycol-di-aldehyde - TPGDA and hexandiol-diacrylate - HDDA), plasticizers (DOS, DOP), ionophores and ion exchangers

(valinomycine, nonactine and KTpClPB), urease, and IsFETs it was developed chemosensors for the determination of K^+ and NH_4^+, as well as a biosensor to monitor the urea level (Munoz et al.,1997). PhI was 2,2'-dimetoxyphenil-acetophenon. Membrane for the sensors to determine the K^+ and NH_4^+ ions included 43.1% and 48.2% aliphatic urethane acrylate (molecular weight 1500), 2.2% and 1.9% of ionophores, 15.2% and 13.4% of HDDA, 38.5% and 34.5% of DOS, respectively. The concentration of PI in both cases reached 2%. Sensor for the potassium determination had sensitivity 56 mV/decade (for 3 months). Similar value (about 59 mV/decade) was achieved for ammonia sensor. Region of linear response was 10^{-1}-$3 \cdot 10^{-5}$ M. The membrane sensor for urea was composed from 76.5% urethane acrylate (molecular weight 1700), 3.5% of urease, 16.2% of TPGDA and 4% of the PhI. Biosensor sensitivity was 63 mV/decade of urea, the linear region was within its concentration of $2 \cdot 10^{-4}$-$6 \cdot 10^{-3}$ M. After a week of operation the sensitivity of the biosensor decreased to 55.8 mV/decade. Draws attention to the biocompatibility of polyurethanes used (no annoying process with the implantation of pieces of polymer in the body of rats).

It was reported (Puig-Lleixaet al., 1999b) about the creation of the biosensor for urea determination with the IsFETs and sensitive membrane based on the photo polymerizable urethane derivatives. To do this, monomers and oligomers used in urethane diacrylate aliphatic, cross-linking agent and 2,2-dimetoxyphenyl-acetophenone served as PI. Urease was as biologically sensitive component which hydrolyzed urea to form carbon dioxide and ammonia resulted to local pH increase. Before applying the polymer the gate surface (silicon nitride) of IsFETs was treated with a solution methacryl-oxypropyl-trimethoxy-silane in ethanol. The content of the composition was as follows: 70% of olygomer, 28% of cross linking agent and 2% of PhI. Approximately 0,15 g of this mixture was homogenized in 0.3 ml of ethanol. Membrane thickness of 100 μm was formed applying 2 ml of cooked mixture to the gate. Mixture was irradiated by UV with a wavelength of 365 nm and 22 mW/cm^2 of power in oxygen-free environment and at the temperature of 20 ˚C. After that the surface was washed with ethanol. Given the fact that the membrane with immobilized biocomponents had insufficient adhesion to the gate surface it was used an original method based on the principle of photolithography. On the edge of the biological membrane and the surface adjacent to it the more hydrophobic polymerizable composition was plotted but without biological components and with the greater adhesion. This area was additional irradiated by UV. As result of this way the central part of the biomembrane remained uncovered by hydrophobic substances and it was polymerized only outdoor areas. It should be noted that the enzyme is not good soluble in the above composition. To solve this problem, tried to pre-dissolve it in water (but at 30% of its content in the composition of the latter is not homogeneous) or glycerol (enzyme dissolved in it worse, but glycerin was better kept in a composition and it was more homogeneous). It was chosen a more appropriate structure of the composition. It was found that at the high content of cross linking agent the membrane is very stiff, quickly exfoliate and at the increasing the water content there is a rapid loss of enzyme activity, membrane is not sufficiently homogeneous and characterized by low adhesion. For further studies was chosen composition which contended 76% of oligomer, 0.5% of cross linker, 22% of glycerol, 1% of the enzyme and 0.5% of PhI. After storage of the prepared mixture for 30 days in the refrigerator the biosensors created on its basis give practically the same answer as that of a fresh composition. The maximum response of the biosensor to urea concentration of $5 \cdot 10^{-2}$-10^{-1} M reaches about 90 mV at pH 5,6. Moreover, at the using of NH_4Cl solution (when the pH depends on the concentration of ammonia and ammonium ion) the biosensor response is linear even at the increasing

concentrations of urea (160 mV to 0.1 M urea at pH 5.6). In additional to the response was more pronounced at 1 M than in 0.01 M NH_4Cl. Time to reach 95% response was about 2.5 min for the concentration of 10^{-4} – 10^{-3} M. Sensitivity of the sensor in a solution of 0.01 M NH_4Cl and pH 5.6 was 58.8±1,2 mV/decade, the region of linear response – 0.04 - 36 mM, for 0.01 M Tris-HCl solution – 35 mV/decade in the field of 1-25 mM. The decline of response during the month was 10%.

2.3 Photo linking in biosensorics

Typically photo-linking prepared polymers are used (Jae Ho Shin et al., 1998; Jobst et al., 1993; Nakako at al., 1986; Barie et al., 1998; Dobrikov & Shishkin, 1983a, 1983b; Dontha et al., 1997; Leca et al., 1995; Nakayama & Matsuda, 1992; Nakayama et al., 1995; Navera et al., 1991). Polyvinyl derivatives such as polyvinyl chloride were widely used at the creation of biosensors (Jae Ho Shin et al., 1998). It was communicated about photo-linked polyvinyl alcohol in aqueous solution for the immobilization of cells of *Arthrobacter globiformis*. PhI in this case is not used. Prolonged exposure to UV light (30 min), poor adsorption and mechanical properties of membranes obtained did not allow them to be widely used in the photo immobilization at the manufacture of biosensors.

At the development of amperometric biosensors for the choline determination the immobilization of cholin oxidize was made in the polyvinyl alcohol containing linked styryl-pyridine groups which served as PhI agent (PVA/SbQ) (Leca et al., 1995). The working and measuring electrodes were made from platinum and calomel electrode was as comparative one. The oxidative potential was on the level of 700 mV. The polymer and enzyme solutions were placed on a platinum disk of the working electrode and were irradiated with UV-source with a wavelength of 254 nm during 45 min. Then the polymer was washed in 30 mM of veronal-HCl buffer, pH 8 at 26 ˚C. It was studied the effect of the polymerization degree and the number of groups on styrylpyrydine on the biosensor response. For this purpose three types of polymer (with a degree of polymerization of 500, 1700 and 2300, and accordingly the number of reactive groups 2.94, 1.31 and 1.06 mol%) were used. The highest sensitivity (21 mA/mol) and the minimum defined limit ($1,5 \cdot 10^{-8}$ M) was obtained for a polymer with a longer chain (and less cross-linking groups). This polymer was selected for further studies. The amount of polymer for the electrode in this series of experiments was 0.22–0.39 mg and immobilized cholin oxidase – 0.7 – 1.7 U (at the activity of 17 U/mg). Next it was studied the effect of enzyme content in biosensor response. If the cholin oxidase content was changed from 0.9 to 2.7 U in 0.3 mg of the polymer it was occurred a slight increase of biosensor sensitivity to choline (20 to 22 mA/mol). The response time was about 10-40 sec. When 0.1 M phosphate buffer (contained 0.1 M KCl at pH 8) were used the determined limit reduced to $5 \cdot 10^{-9}$ M, however, narrowed the region and a linear response - $4 \cdot 10^{-8}$ – $4.5 \cdot 10^{-5}$ (vs. $1.5 \cdot 10^{-8}$ - $4.5 \cdot 10^{-5}$).

It was studied the effectiveness of immobilization of butyryl cholin oxidase in the PVA/SbQ-matrix in the comparison with the BSA-matrix cross-linked with glutaraldehyde (Wan et al., 1999). The polymer membrane was manufactured as follows. PVA/SbQ (45 mg) was mixed with the enzyme (5 mg) in phosphate buffer (50 mg, 1 mM, pH 8.0). These mixtures (0.5 ml) were applied to the gate of the IsFET and then irradiated with UV during 25 min. The greatest response of both biosensors to butyryl cholin was found when the phosphate buffer (pH 8.0 at the concentration of 1 mM) was used. Region of linear responses of biosensors measured in dynamic regime was 0.2-1 mM and 0.2 – 5.8 mM and the calculated KM achieved 2 mM and 3.8 mM for BSA- and PVA/SbQ-membranes,

respectively. When storing the biosensor with PVA/SbQ-membrane in the dry state and in the dark at 4 ˚C for 9 months the fall of its response was 20% (similar to the decline in storage in a phosphate buffer at pH 8 in the same conditions was achieved at 1 month). For the biosensor based on BSA-membrane the similar declines of the responses were through 7 and 42 days when it was stored in a dry state and in the buffer, respectively. The field of the determination of such organophosphorus pesticide as trichlorphon was similar for both types of biosensors and amounted to 10^{-3}–10^{-6} M

Navera et al. (1991) reported about the development of the acetylcholine biosensor using carbon fibers. Acetyl cholinesterase and cholin oxidase were co-immobilized in polyvinyl alcohol with a stiryl pyrydine as cross linking agent. Duration of response was 0.8 minutes and the linear region was within 0,2-1,0 mM.

Jobst et al. (1993) created oxygen amperometric biosensor for the application in vivo condition. Selective membrane was made from the poly-N-vinilpirolidon cross linked with 2,6-bis-(4-azidobenziliden)-4-methylcyclohexanone (total 3%) under UV irradiation. For 10 sec 95% of the response is realized and its value in the presence of dissolved oxygen in the water reached about 200 nA.

The biosensor based on the IsFET for the determination of a neutral lipids [34] was developed on the sensitive membrane obtained photo-crosslinking polyvinylpyrrolidone (PVP), 4,4 '-diazidostilben-2,2'-disulfonate sodium (0,1 g of cross-linking reagent in 100 ml of 10% aqueous solution of PVP). To 200 ml of this solution 15 mg lipase and 10 mg BSA were added. This mixture was applied to the IsFET gate, centrifuged at 3000 rev/min for 2 min and irradiated with a mercury lamp during 5 min. Then the mixture was treated during 15 min with a solution of glutaraldehyde at 4 º C and finally it was kept in 0.1 M solution of glycine (4 ºC). The chips were stored in a buffer solution at 4 ºC. Linear fields of responses were as follows: for triacetin - 100-400 mM, tributylin - 3-50 mM and triolein – 0,6-3 mM. The minimum detectable concentration of the last was 9 mg/ml. Decline in response for 3 months was 12% only.

At the development of immune biosensors based on surface acoustic waves to detect a specific protein (urease) as photo-crossing agent served bovine serum albumin (BSA) modified aryldiazirine (Barie et al., 1998). Aryldiazirine absorbs light with a wavelength of 350 nm and forms a highly reactive carbenes, which are preferably interact with the C-H, C-C, C=C, N-H, O-H or S-H groups. The surface of the transducer was sialinized by dimethylamino-propyl-ethoxy-silane, then coated with a polyimide film (thermal polymerization mixture *p*-phenylenediamine and 3,3',4,4'-biphenyl-tetracarbocyclic dianhydride) or parilene C (poly (2-chloro-p-xylene). Then, on the surface it was applied the mixture of triftor-methylaryl-diazirine BSA (T-BSA) with dextran and its was irradiated by UV-source (0,7 mW/cm_2, the main emission 365 nm). For the glass surfaces, passivated by parilene the optimum ratio was: 75% T-BSA and 25% dextran at the irradiation time of 45 min. The density of dextran on the surface was 1 ng/mm_2. The special peptides – antibodies to urease were linked to the carboxylated dextran with a mixture of N-hydroxysuccinimide and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in 1:4 ratio (passivating layer was polyimide, the operating frequency - 379, .43 MHz, the loss during the passage - 4.89 dB). It was received a response to urea at concentrations of 15-500 μg/ml with a maximum shift of the oscillation frequency transducer 110 kHz.

BSA derivatives were used for cross-linking antibodies to planar optrods (Gao et al., 1995). On the surface of the waveguide (TiO_2/SiO_2) a mixture of 3-(trifluoromethyl)-3-(m-izotiocyanophenil) diazirine derivative of BSA and $(Fab')_2$ fragments of antibodies (4:1) was

placed and then it was irradiated with UV-source (0,7 mW/cm_2, 20 min) Immobilized antibodies were specific to the prostate antigen. The density of immobilized antibodies was 16.8 fmol permm_2 or 1.05 μg per chip. Biosensor sensitivity reached 0.35–3.5 μg of protein per chip. The biosensor had a low non-specific response. Its regeneration was curried out by treatment with glycine buffer (pH 2.3). When storing the biosensor in the presence of 0.5% BSA, and 4 ˚C during the month there is no significant activity decrease.

2.4 Application of photo polymerisable matrix at the creation of potentiometric enzymatic biosensors

Early (Arenkov et al., 1994a; 1994b; Levkovets et al., 2004; Nabok et al., 2007; Starodub et al., 1999a; 1999b; 2000a; 2002a; Starodub & Starodub, 2002; Starodub, 2006; Starodub et al., 2008) we have developed prototypes of the enzyme- and immune biosensors based on the IsFETs and the electrolyte insulator semiconductors (EIS) structures. Both types of the biosensors are perspective for use in different fields, in particular, medicine, biotechnology and environmental monitoring. Nevertheless, before start of their wide manufacturing there is necessary to optimize the procedure of biological material immobilization on the transducer surface. In generally it is the main problem of biosensorics and for it's solving a lot of different approaches including pure physical, chemical and hybrid physical-chemical methods were proposed (Pyrogova & Starodub, 2008; Starodub et al., 1990; 1995; 1998; 1999c; 2001; 2005). All these methods are directed on more effective fulfillment of the main practice demand which concern the achievement of maximal level of residual activity of biological molecules and exposition of their active centers toward solution, simplification of procedure of immobilization and its combining in unique electronic cycle of transducer manufacturing, preservation of high level of biosensor response during its storage and working, etc.

The application of the liquid polymerisable compositions (LPC) on the basis of monomer-olygomeric substances at the biological membrane creation may be considered as perspective approach directed on providing above mentioned practice demands. These compositions give possibility to form sensitive membranes with adjustable physical-chemical and mechanical abilities without strong temperature and chemical destructive effects on biological molecules. Among the most wide dispersed LPC it is necessary to mention a number of monomeric and olygomeric acrylate compounds (acrylic, metacrylic acids their ethers and derivatives) as well as urethane olygomers and vinyl copolymers (sterol, vinyl acetate, vinylidenchloride, vinylpyrrolidone and others). At the varying of chemical origin and concentration of some components there is possibility to regulate a lot of parameters of biological membranes obtained on the basis of these components (Rebrijev, 2000; 2002; Rebrijev et al., 2001; 2002a; 2002b; Rebrijev & Starodub, 2001; Starodub & Rebrijev, 2002; 2007; Starodub et al., 2002b).

The use of the LPC in biosensors supposes that they should be characterized by number of indexes, namely: they should be non-active concerning biological substances, permeable in respect of determined analytes, as well as have defined hydrophobic-hydrophilic balance and sufficient level of adhesion to the transducer surface. The liquid photopolymerisable composition (LPhPC) causes special interest in biosensorics. Although it's wide application is restricted by the practice demands above-mentioned. As a rule at the biosensor creation the influence of supported substances on the biological materials is not special observed. Usually the excess of biological material is taken and for the estimation of its state the non-direct approaches are used, namely: the determination of biosensor response, the rate of

product formation and others. At the same time the change of structure of biological molecules at the creation of biochips or during their preservation reflects disproportionately on the intensity of response and lifetime of biosensor work. Moreover at the multi-layer immobilization of biological material the inner layers may work with the small productivity in comparison with the external ones due to the diffusive restrictions. That is why the main purpose of this work was the elaboration of content of the LPhPC, which is characterized by number of abilities in concordance with the biosensorics demands in respect of above mentioned and some additional ones: simplicity of immobilization procedure and homogeneity of formed membrane. To optimize the conditions of the enzyme including in the LPhPC the absolute level of residual activity of the immobilized molecules was determined and the principal factors affected on this level were characterized.

In experiments it was used: urease from soybean with activity of 200 u/mg (Sigma, USA), GOD from *Penicillium vitale* with activity of 160 u/mg (Kamenskoe distillery, Ukraine), horse radish peroxidase (HRP) of type VI with activity of 275 u/mg (Sigma, USA).

N-vinylpirrolidone (VP) was obtained from "Aldrich" (Germany). 2-hydroxy-2methyl-1-phenylpropan-1-on (Darocure 1173, λ_{max} = 310-350 nm) from "Ciba-Geigy", Switzerland) served as PhI. Monomethacrylate ether ethyleneglycol (MEG) was produced by "BASF" (Germany) and olygocarbonatediethylenglycolmetacrylate (OKM-2) by AOOT "Korund" (Russia). Olygouretane metacrilate (OUM-1000T or OUM-2000T) was synthesised according to (Masljuk & Chranovsky, 1989).

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 (Starodub et al., 1990). 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. Polymerisation of this mixture was curried out at the effect of the UV radiation in vacuum conditions (0.1-0.2 mm of mercury). As source of the UV it was used lamps: LUF-80-04 (λ_{max} = 300-400 nm, intensity of light on the irradiated surface – about 2.6 Watt/m^2) and DRT-120 (λ_{max} = 320-400 nm, intensity of luminous flux about 12.5 Watt/m^2).

The homogeneity of composition and obtained polymer was determined by visualization, i.e. the absence of visible disseminations at microscopy was taken as maximal level of this index and was marked as (++). Adhesion abilities of the formed polymer were non-direct appreciated on the assumption of time being membrane on the transducer surface without its peeling at the immersion of chip into buffer solution. The extreme positions, i.e. immediate peeling of membrane was marked by (--) and its attaching during two month – by (++). In case of the determination of the residual enzyme activity the LPhPC was presented as two-component mixture containing VP and PhI at 98 and 2 g/100g of concentration, respectively. Then, to 50 µl of this mixture and 20 µl of the enzyme solution was added at the shaking and water was removed in 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 was 11 and 4 min at the application of LUF-80-04 and DRT lamps, 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 of 5.5, 7.0 and 6.0 in case of the determination of activity of GOD, urease and HRP, respectively.

It is necessary to mention that at the obtaining of calibration curves the VP, PVP or intermediate products of these substances (depends on duration of irradiation or method of analysis) were added to the analyzed samples. The some details of experiments are given in the text below.

According to the preliminary investigations as main component of the LPhPC it was taken VP as substances with appropriate hydrophilic-hydrophobic balance. The optimal contents of the enzymes and PhI were 3 and 2g per 100g of LPhPC, respectively. Primarily MEG was used as cross-linking polymers. The results of choosing optimal variant of the LPhPC in respect of homogeneity of the obtained polymer, its adhesion to transducer surface and biosensor response are summarised in Table 1.

Applying the above LPhPC and immobilized GOD on the transducer surface it was created biosensor for glucose level control (Fig. 1). It had the following characteristics: linear response region in frame of 0.1 - 10 mM, the slope of the curve 30 mV/pC and response time during 10 -15 min. Km values for GOD immobilized in photopolymer material is 3.1 mM. To calculate Km used graphical method of inverse coordinates. In the literature there is information about the positive experience of the introduction of the LPhPC glycerol, which was injected together with enzyme in a hydrophobic matrix. We also carried out attempts to introduce GOD in the chosen composition of LPhPC using glycerol (in an amount which was 5, 10 and 20 of wt.%). However, it turned out, this led only to a deterioration of the homogeneity of composition and adhesion of the polymer as well as to reducing the latter to the surface of the transducer. So we abandoned the use of glycerol in LPhPC.

Thus, the obtained LPhPC due to its properties for ease of manufacturing and process of biomaterial immobilization may be included in extended technological stages of photolithographic manufacture of semiconductor structures. The created on this basis biosensor may have the characteristics needed for use in laboratory, clinical, food and biotechnology practice.

VP, mas.%	MEG, mas.%	OKM-2, mas.%	OUM-1000T, mas.%	Homogeneity		Adhesion of membrane to ISFET surface	Response on 10 mM GOD**
				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***	+	+	++	57

Table 1. Some characteristics of the LPhPC based on VP*. *Quantity of PhI in all LPhPC was 2 mas%. ** In 1 mM sodium phosphate buffer, pH 7,0. *** Instead of OUM-1000T it was used OUM-2000T

In literature as a rule, the degree of decrease in activity of biological material in the process of immobilization is not special considered. For the state of biological structures it is using indirect methods such as measurement values the sensor response, speed of formation of

different substances, etc. It should be noted that for the immobilization is usually initially taken excess of biological material. However, increased activity of enzymes in the selection of optimal conditions for this process or its decrease in functioning and maintaining biochips disproportionately affects on the efficiency of the measuring device (the intensity of his response, duration of work etc.). Moreover, in most cases the biological material is immobilized often by multilayer and thus the inner layers operate with lower productivity due to diffusion limitations.

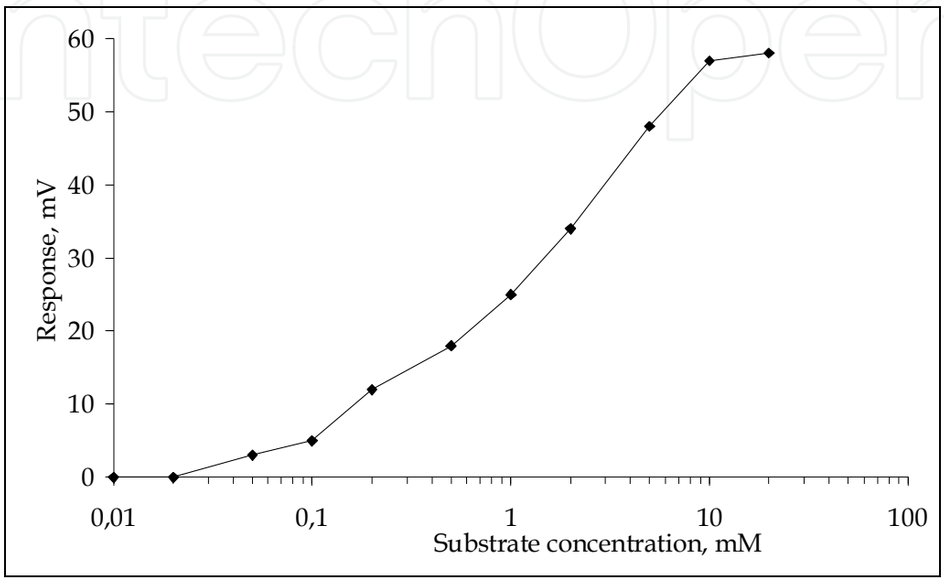


Fig. 1. Response of biosensor with the immobilized GOD (substrate – glucose). Measurements were made in 1 mM of sodium-phospate buffer, pH 7,0.

That is why, the next experiments were fulfilled for the estimation of the absolute level of residual activity of immobilized enzymes, as well as the main factors influencing this level, to determine the optimal conditions for the inclusion of enzymes in photopolymer membrane. For this purpose the enzymes immobilized in LPhPC based on VP. The obtained on this basis polymer was water soluble, so after the dilution of its in buffer solution can there is possible to study the activity of immobilized enzymes.

Fig. 2 presents the results of changes of GOD activity at the including into PVP matrix depending on the source of UV radiation. These data suggest that the decreasing activity of the enzyme occurs to a greater extent when as a source of UV radiation it was used LUF (32.45%) than DRT lamps (37.25%), $p < 0.05$. The presence of VP and PVP in GOD solution made no significant influences on the level of activity, which can serve as an indirect indicator of chemical inactivity of VP and obtained polymer in respect of the enzyme.

It is known that immobilization of biological material is usually preceded by dissolving it in buffer solutions. However, mixing composition, which is able for photo polymerization, with a buffer solution, usually, leads ultimately to a deterioration homogeneity system and mechanical properties of the resulting polymer, due to the presence of salt ions in the system. Therefore, interest was to find out the possibility of eliminating this effect by replacement of buffer solution on distilled water when the preparing compositions contained biological material. First of all, it was necessary to

establish the impact of replacing the buffer solution on distilled water for preservation of enzyme activity in the polymer. Consideration of the data is shown in Fig. 2 (UV irradiation LUF for 11 min.) It was shown that the replacement solvent has not affect on the level of residual enzyme activity in the membrane. This was the reason to exclude in these studies the use of buffer solutions with the introduction of the enzyme in the photo polymerizable composition.

The irradiation of the GOD solution (10 mM sodium phosphate buffer, pH 5.5 over time, which corresponds to that given during the course of polymerization, i.e., 11 and 4 min for different powers of UV sources - LUF and DRT) does not significantly affect on the change of activity of the enzyme studied.

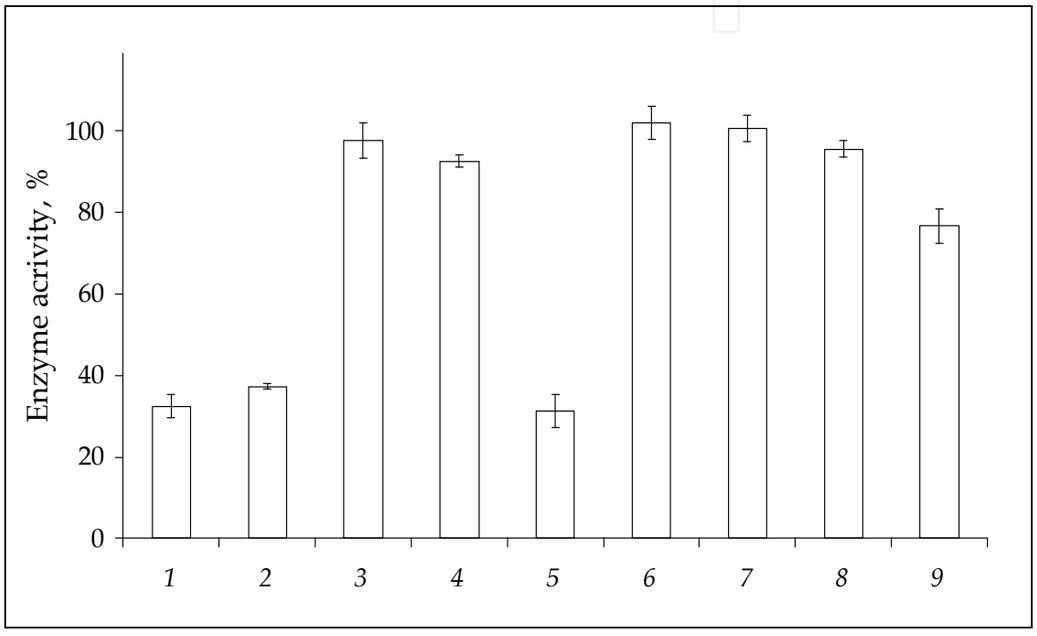


Fig. 2. Residual activity of GOD under different conditions of preparation of membranes. Where: 1, 2, 5 - photo polymerization in VP, 3 - in a mixture of solutions of GOD and VP, 4 - a mixture of solutions of PVP and GOD, 6, 7 - UV-irradiation of buffer solution of GOD, 8, 9 - mixture of solutions of GOD and PhI in glycerin (1, 5, 6, 9 - LUF irradiation; 2, 7 - irradiation of DRT; 1, 2, 3, 4, 8, 9 – GOD was previously dissolved in water, and 5 – GOD was previously dissolved in 10 mM sodium phosphate buffer solution, pH 5.5.

It was interested to study the effect on the GOD activity of another component LPhPC - PhI. For this purpose it was necessary to take into account that the used 2-hydroxy-2-methyl-1-phenylpropan-1-one as PhI is insoluble in water. To this end in LPhPC was used 2% solution (mas.) of PhI in glycerin, which in turn dissolves in water.

As shown in Fig. 2, when entering GOD (water solution) in this composition noticeable change in enzyme activity is not observed. At the same time UV-irradiation of this mixture (source - LUF) leads to a reliable ($p < 0.005$) lower enzyme activity, representing 76.7% of the initial level. However, it is established that at the use of DRT and LUF for photo immobilization the residual activity is according to peroxidase 41.5% and 44% and for urease - 21% and 16.5%, reliable data, $p < 0,05$ (Fig. 3). Conditions of the experiment were the same as in case of GOD immobilization.

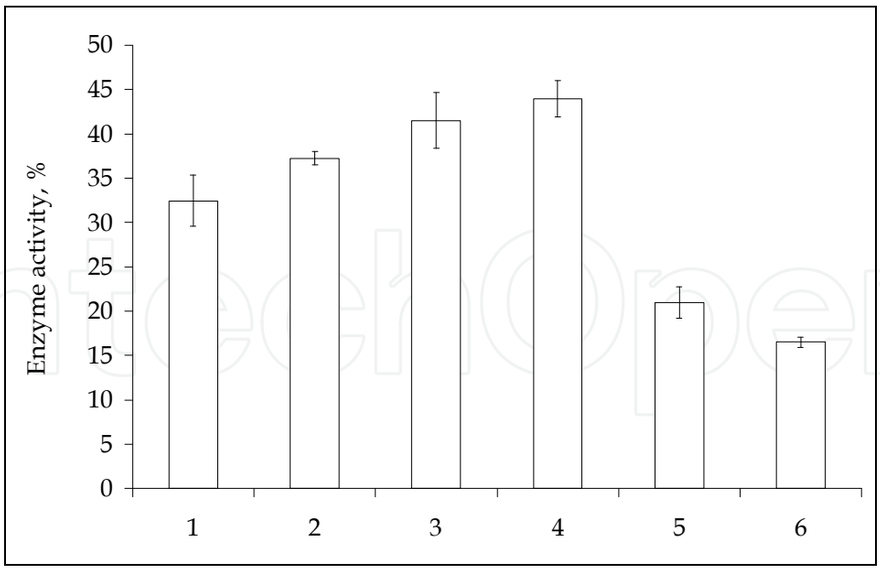


Fig. 3. Residual activity of GOD (1, 2), peroxidase (3, 4) and urease (5, 6) in photo polymerizable matrix. Source of irradiation: LUF – 1, 3, 5 and DRT – 2, 4, 6.

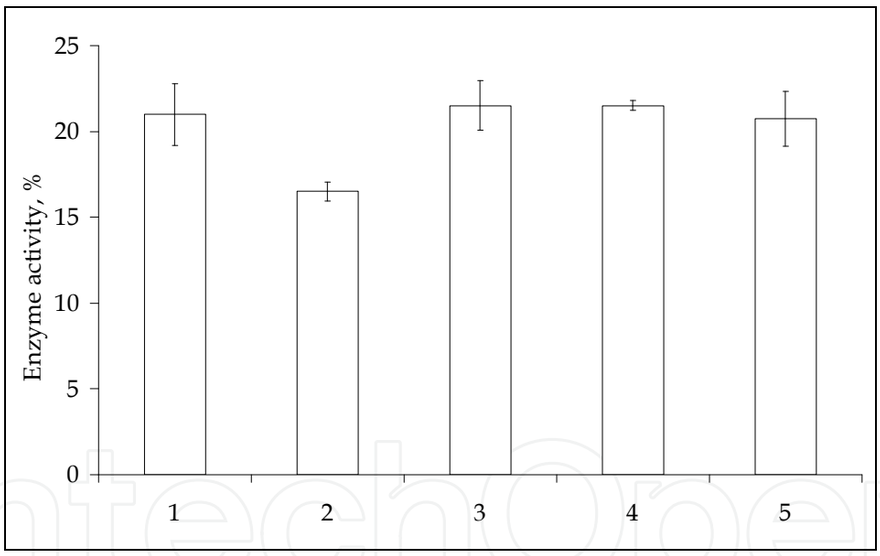


Fig. 4. The level of residual activity of urease after photo immobilization. Where: 1, 2 – without filter for UV; 3, 4 – with application of glass filter; 5 – in condition of low temperature (-8 ^{0}C). Source of irradiation: LUF – 1, 3, 5 and DRT – 2, 4.

Unlike GOD and peroxidase urease reveals itself as the low stable enzyme. The fall of its activity is due, mainly, oxidation sulfhydryl groups present in the active center. This enzyme is subsequently used for working out optimal conditions for immobilization. In addition, interest was to determine the influence of UV radiation of different wavelengths on the amount of residual enzyme activity. For this purpose, the short-wave area up to λ = 300 nm was cut off by a filter (glass). At the using glass (3 mm thick) as the UV-irradiation filter to 300 nm and without it's the enzyme activity in the mixture after irradiation LUF did not change (Fig. 4). However, note that in similar conditions DRT-irradiation the enzyme

activity significantly increased ($p < 0.001$), reaching some of the value that was registered using the LUF-irradiation. This experimental fact, most likely due to the fact that short-range (220 - 280 nm) lamp DRT, which has great energy, influences on urease. At the same time, irradiation of LUF with λ_{max} 300 - 400 nm, when the radiation is almost entirely absent in the 220 - 280 nm using a glass filter, did not affect on the activity of the enzyme. Thus the measured power of UV radiation of DRT (220 - 280 nm) was equal to 12 W/m², which is 60% of the energy range 300 - 400 nm. Data about the effect of low temperatures (-8 ° C) on urease activity presented in Fig. 4. Given the fact that the freezing point VP is +13 ˚C, it should be noted that the photo polymerization at -8 ˚C was carried out in solid phase. Apparently, lowering the temperature of polymerization mixture to -8 °C is not made definite influence on the residual activity of urease.`

To investigate the dependence of the residual activity of urease from time of influence of LUF illumination it was chosen the next time range: 220, 330, 440, 660 and 990 sec. It was found that the enzyme activity decreases after the most exposure for 300 - 420 sec. (Fig. 5). Typically, kinetics process of the polymer solidification had S-shaped character. To measure the degree of polymerization the spectroscopic studies of irradiated RFPK were carried out by infrared spectrophotometer SP-300S Philips with the various time of intervals. The degree of conversion was judged by peak area with a maximum range of 1640 cm^{-1}, which corresponds to the double carbon-carbon bonds in VP that quantitatively reduced in a polymerization composition in the comparison with the relatively quantified not variable carbonyl VP group, which has a maximum peak at 1700 cm^{-1}. The drop in enzyme activity correlates with the polymerization matrix.

It is well known that to preserve the active center of urease during immobilization using blocking its substrate analogs that do not split, for example, thiourea. Thiourea molecule is similar in structure to urea and a urease competitive inhibitor. Introducing thiourea in a mixture and analyzing the activity of the enzyme by the above mentioned method, its impact can not be set because it is constantly present in solution. To avoid this, it was used the following approach. It lies in the fact that the first LPhPC consisting of Oum-2000T - 10 wt. %, VP - 88 wt. % and PhI - 2 wt.% was prepared.

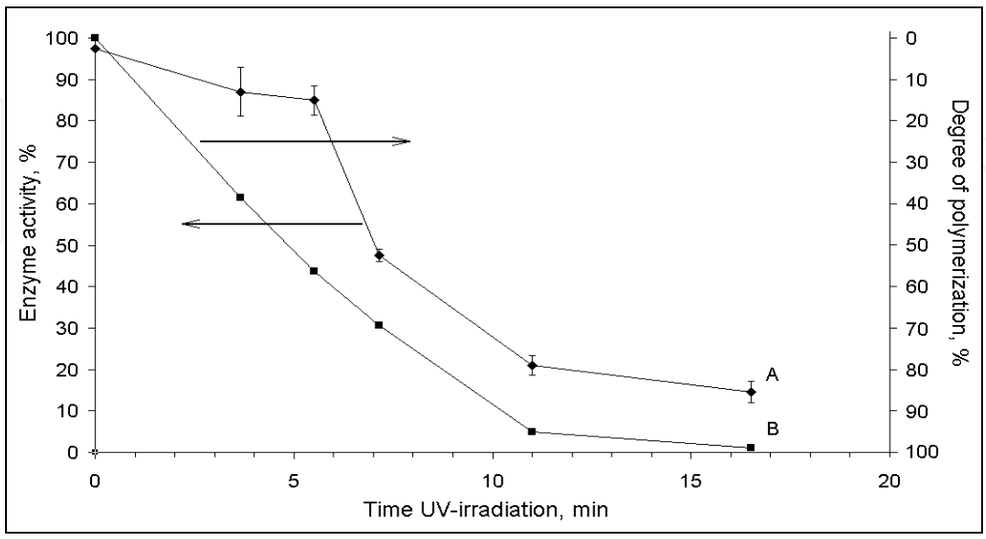


Fig. 5. Dynamics of changing in urease activity in dependence on time of UV irradiation by LUF lamp.

OUM-2000T - is a urethane oligomer with a molecular mass of 2800 with terminal methacrylate groups, i.e. tetra functional compound that performs role of cross linking reagent in this photo polymerizable compositions. Thus, at the photo solidification of this composition the strong three-dimensional polymer is formed, but very flexible. In LPhPC the enzyme solution was injected and this mixture after photo solidification formed the strong elastic film with the thickness of 0.1-0.15 mm. Also, the control film was prepared that does not contain thiourea. Then within two days the films were washed from thiourea. Urease activity was calculated per unit surface of the film. Activity of the enzyme in control films was taken as 100%. The results presented in Fig. 6 shown that at 0.5% (mas.) of the initial contents of thiourea in LPhPC the residual urease activity increases on 11,3% ($p < 0.05$). At the same time increasing the thiourea content in the composition up to 1% stabilized the enzyme in less degree.

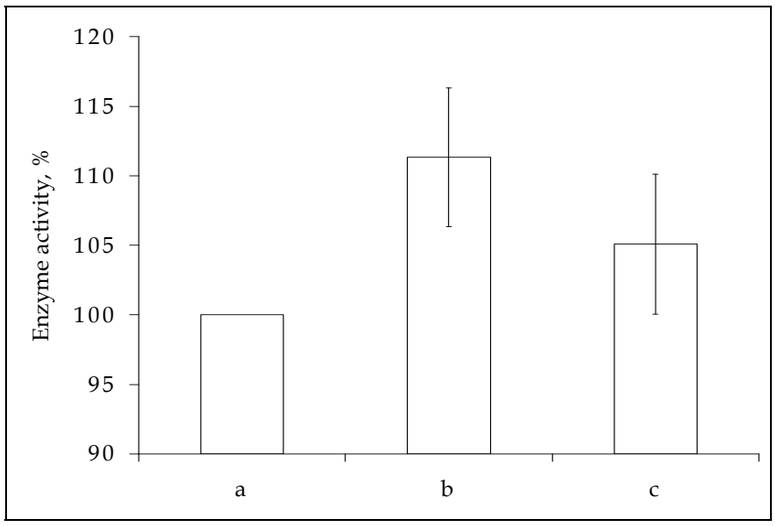


Fig. 6. Influence of thiourea content in phtopolymerizable composition on the activity of the immobilized urease. Content of thiourea according to mass:: *a* – 0 %, *b* – 0,5%, *c* – 1 %.

It was stated that the urease activity decreased in LPhPC at its preservation (at - 4 ˚C). Trough two months this decreasing reached 15% ($p < 0.05$) (Fig. 7) then this index continued to decline and after six months the reduction was a few less than half (47%) of fresh compositions ($p < 0.005$). At the same time while maintaining the urease in photopolymer matrix (with PVP), a marked decrease in its activity during the two months was not observed. Only after 6 months it was indicated the significant decrease in its activity, which was approximately 30% ($p < 0.01$). Saving GOD over six months in the PVP-matrix leads to a decrease in its activity about 23% ($p < 0.005$).

When the low (-35 - -50 ^{o}C) temperature was used for the polymerization the level of residual enzyme activity increased up to 50% at -50 ^{o}C in comparioson with the polymerization in ordinary (20 ^{o}C) conditions ($p < 0,002$). The required low temperature was achieved using liquid nitrogen (Fig. 8).

Therefore, it was proposed a method of determining absolute enzyme activity during immobilization in a polymer matrix and it was characterized the changes of enzyme activity (GOD, peroxidase, urease) at photo immobilization. The main attention was paid to the dynamics of changes of enzyme activity in the process of photo polymerization when UV

irradiation was used. The needed conditions for increasing the activity of enzymes at the immobilization and at the storage prepared membrane were chosen.

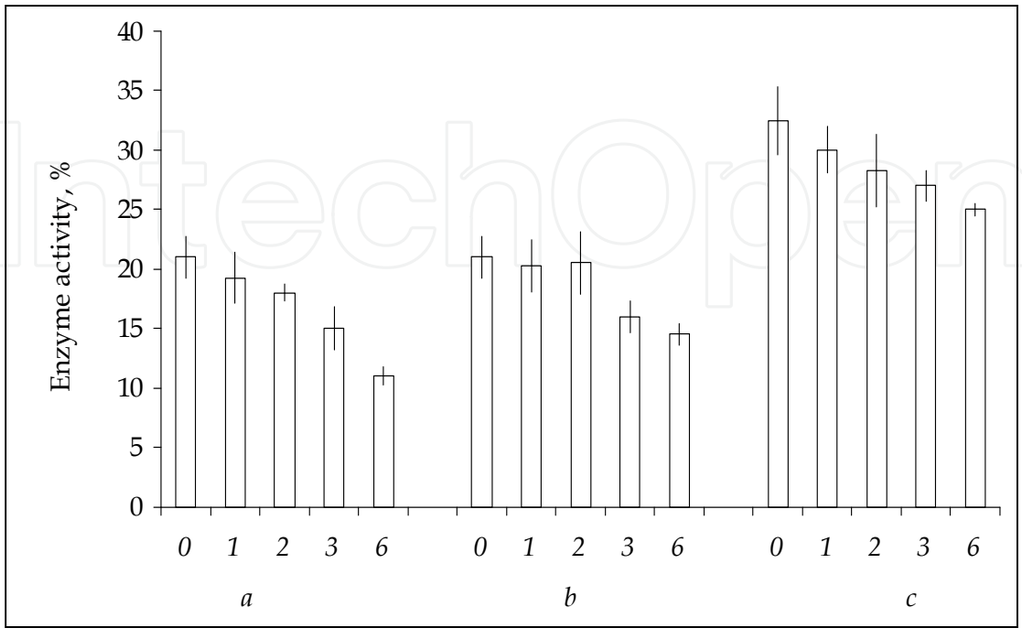


Fig. 7. Dynamics of changes of enzyme activity at the preservation (figures under the columns – quantity of month). Enzyme used: *a, b* – urease, *c* – GOD. Preservation in non polymerised composition (a) and in PVP matrix (b, c). Irradiation – by LUF lamp.

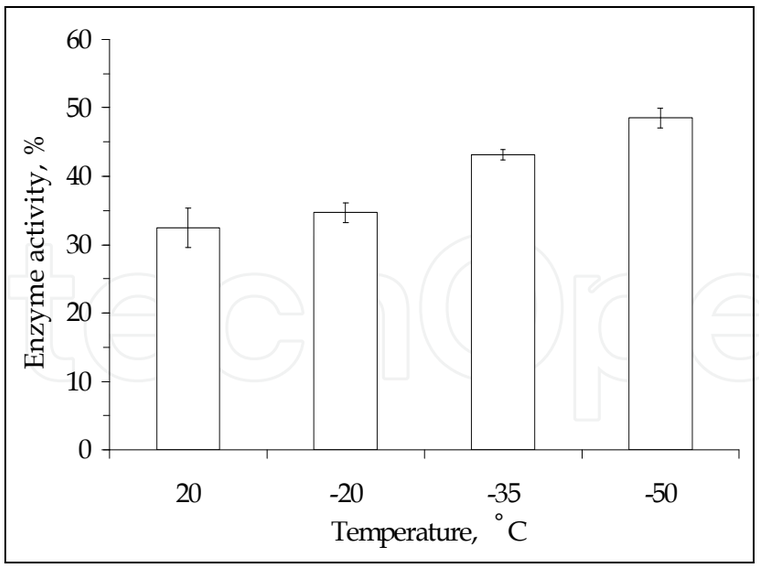


Fig. 8. Effect of temperature during LPhPC polymerization on the residual GOD activity.

2.5 Characterization of work efficiency of urea biosensor with LPhPC

This enzyme was chosen as such which has a much low stability in the comparison with others ones mentioned above. Upon the addition of urea in the test cell the potential at the

IsFET gate decreases as result of pH growth. Noticeable changes are found only during 0.5-3 min after substrate adding. Then, trough a few minutes decreasing voltage signal stops and it goes to the plateau. With increasing concentration of urea the biosensor response time decreases. For example, the duration of the analysis of 0.1 mM of urea solution is 10 min. and at 1 mM of substrate concentration - 4 min. Dependence of the biosensor response on the urease content in the composition is illustrated in Fig. 9, on which is shown that the greatest response observed at the presence in its of 3% of enzyme (mas.). The graph shows that there is a linear relationship between the content of the enzyme in the composition and the biosensor response. In accordance with this relationship it can be concluded that further increase the enzyme content in the composition biosensor response could be larger, and therefore the higher sensitivity of the sensor. However, the attempts to further increase of the enzyme content in the composition led to a sharp deterioration in both its homogeneity and solidity derived from its polymer with immobilized enzyme.

The work of the IsFET based biosensor depends not only on the acidity of the medium and also its ionic strength, but effect of first is much stronger than the second one. It is well known that the work potentiometric biosensors depend on the buffer capacity of solution, which eliminates local changes in pH under the gate region. The developed biosensor showed the largest response in 1 mM sodium phosphate buffer (Fig. 10). However, it should be noted that even at 10 mM buffer, the urea biosensor response was quite significant if the substrate solution was present in concentrations of not lower than 0.5 mM. It is worth noting that the concentration of urea in the blood serum of healthy individuals is 2.50 - 8.33 mM and it increases to 50 - 83 mM in the case of kidney failure as a result of various diseases. So enzymatic biosensor based on the proposed biological membranes can be successfully used for measuring the concentration of urea in the blood without its additional dilution that distinguishes this biosensor from others early proposed (Arenkov et al., 1994a; 1994b; Levkovets et al., 2004; Nabok et al., 2007; Starodub et al., 1999a; 1999b; 2000a; 2002a; Starodub & Starodub, 2002; Starodub, 2006; Starodub et al., 2008).

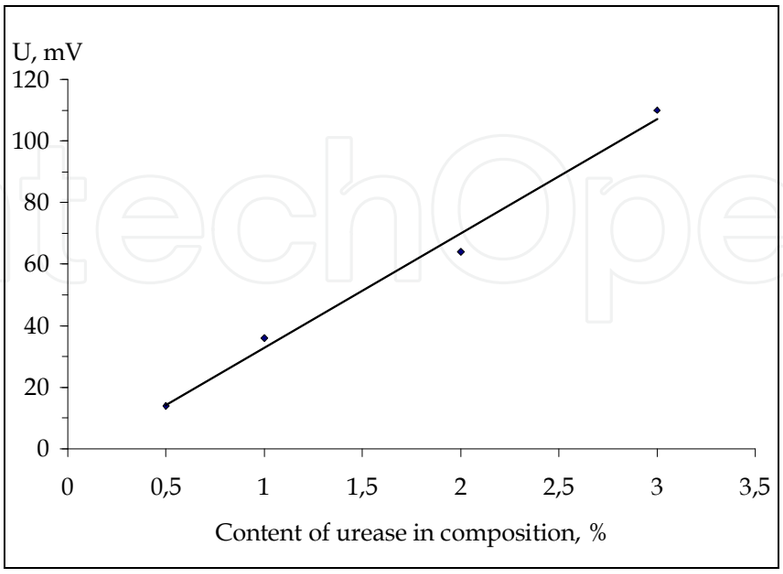


Fig. 9. Dependence of the biosensor response on urease content in the composition.
Conditions of measurement: 1 mM of sodium-phosphate buffer, pH 7.3 and 5 mM urea.

Dependence of biosensor response on temperature (Fig. 11) shows that with its increasing from 28 to 41 °C the value of response increases by 15%. Similar data on the dependence of the sensor response on the temperature were obtained by us when the sensitive membrane was cross-linking enzyme with the protein carriers by glutaraldehyde (Soldatkin at al., 1993).

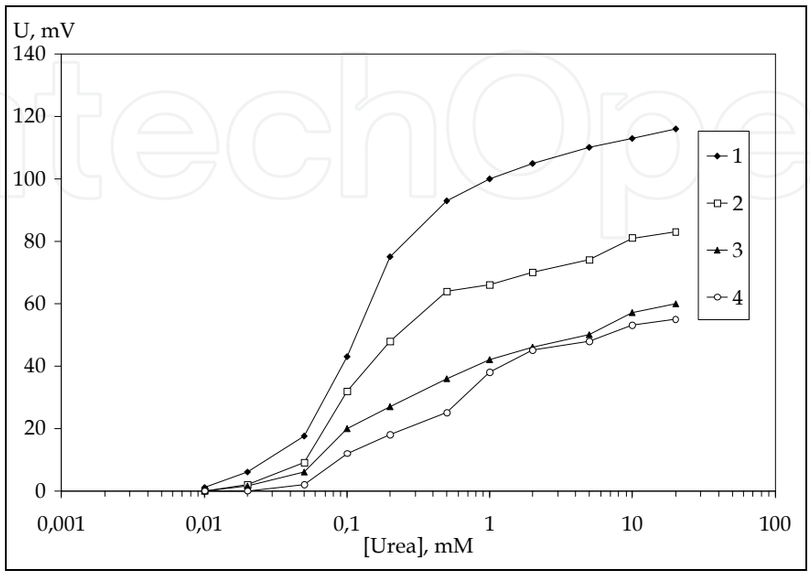


Fig. 10. Dependence of biosensor response on buffer capacity of the analyzed solution. 1-4 – concentration of sodium-phosphate buffer: 1; 2; 5 i 10 mM respectively.

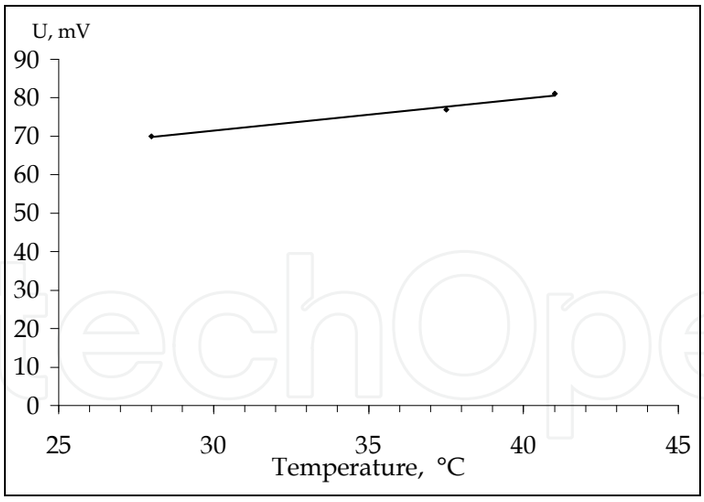


Fig. 11. Dependence of the biosensor response on the temperature. Conditions of measurements: 2 мM sodium phosphate buffer, pH 7.3; 2 mM urea.

It is well known that the optimum pH for urease is at 7.4. Therefore, studying the dependence of sensor response on pH it was conducted in a range from 5.5 to 8.5 at intervals of 0.5. In these experients polimiks-buffer (containing 2.5 mM citric acid, tris hydroxymethyl aminomethane, borax and potassium dihydrophosphate) that supports the buffer capacity in the pH range from 4 to 9. According to the data shown in Fig. 12, the maximum response

in this case is achieved when the pH level was in frame of 6 - 6.5. Properties of urease immobilized probably a little different from those which are characteristic for the free enzyme.

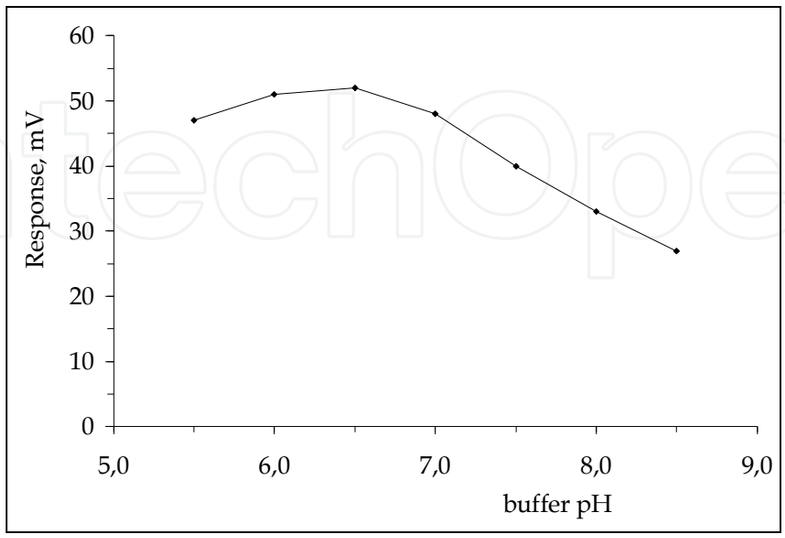


Fig. 12. Dependence of urea biosensor response on buffer pH (1 mM of urea, 10 mM of polymix buffer.

For biological fluids is characterized by the presence of some salts in different concentrations, so it was important to determine the dependence of biosensor response on ionic strength solution of NaCl (basic salt contained in biological fluids). As follows from Fig. 13, increasing concentrations of NaCl in the analyzed solution leads to a decrease in biosensor response for urea (1 mM in 10 mM sodium phosphate buffer, pH 7.0). At NaCl concentration of 300 mM falling response is about 50% but at the next increase of salt concentration up to 500 mM falling response practically does not observe.

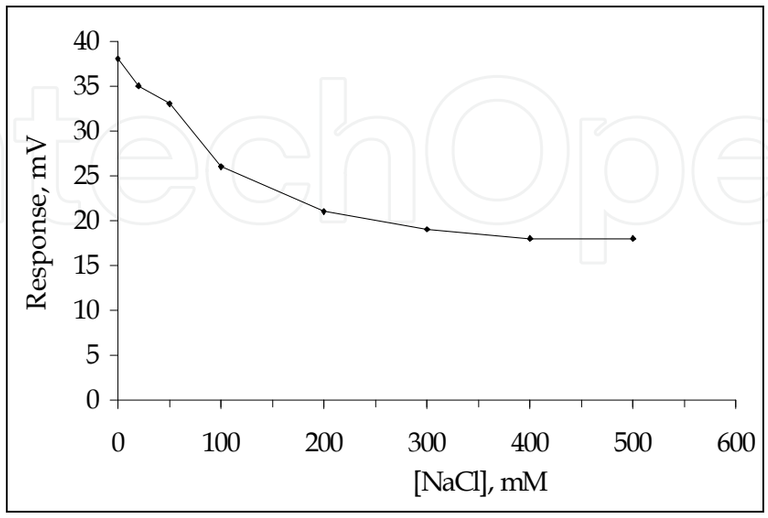


Fig. 13. Dependence of biosensor response on ionic strength of solution to be analyzed (1 mM of urea, 10 mM of sodium phosphate buffer).

In order to verify if the biosensor could be used in real conditions for analysis of human serum the measurements were conducted by both the developed biosensor and a standard colorimetric method using nessler's reagent. The serum blood was preliminary diluted by 10 mM of sodium phosphate buffer (pH 7.3). The data presented in Fig. 14, indicate a high level of coincidence of results obtained by both methods. But for a single measurement differences in test results by these methods were in the range 15-20%.

The special interest at the development of biosensors always the question is aroused about possible time of them operations. It was shown that the intensity of the response of the developed biosensor gradually decreased in course of 40 days. Moreover, during this period reduce of the intensity of response was 20% (Fig. 15). This indicates the possibility of significant extension of time functioning biosensor. As it was mention above urease contains in the active center sulfhydryl groups, which a lot of what determines the loss of enzyme activity over time. The latter are evident in the case of chemical modification or partial denaturation of the enzyme at the formation of biosensor membranes. Under the conditions of experiment the formed enzymatic membrane slowly loses its activity and life can be above or even higher limits.

In the developed photo polymerizable composition enzyme is probably in a stabilized condition. This confirmed by data about the studying responses of the biosensors, biological membranes of which were obtained from the freshly composition and prepared from one preserved in a dark place at 2 °C for 46 days. According to results shown in Fig. 16 the differences in the intensity of responses of biosensors that used these membranes are absent. These data suggest the possibility of long storage of the finished compositions without significant decrease in enzyme activity. In addition, this experimental fact indicates the promising application of compositions in industrial manufacturing sensors with immobilized urease. It seems that pre-prepared photo polymerizable composition can be used for a long time in the process fo the photolithographic formation of biologically active membrane of biosensors. Moreover, this process may be continuing technological production of IsFET using basic approaches of integrated electronic technology

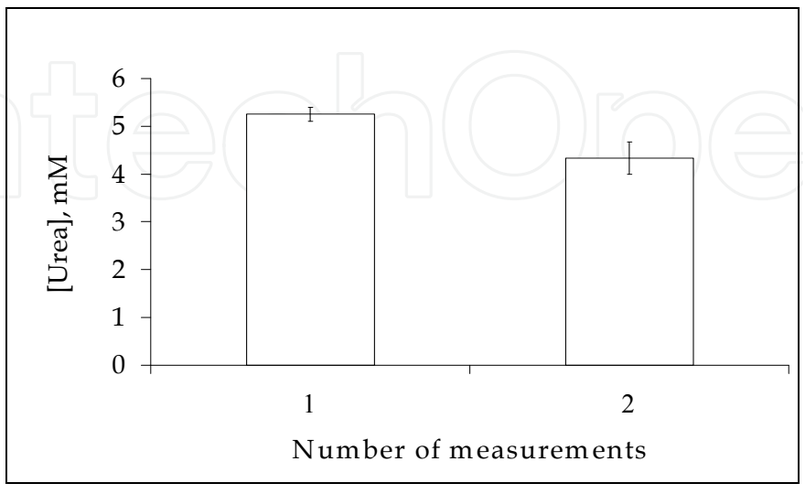


Fig. 14. Determination of urea in the serum blood by the colorimetric method (1) and by the developed biosensor (2).

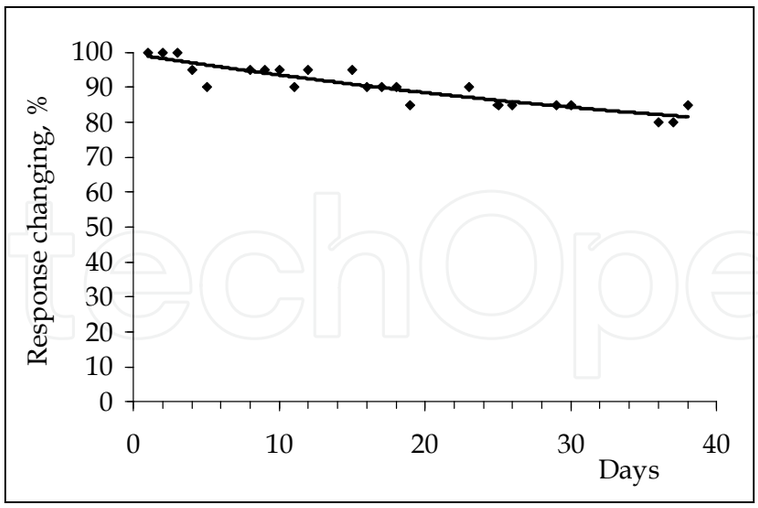


Fig. 15. Changing of response level of urease biosensor during time of its functioning.

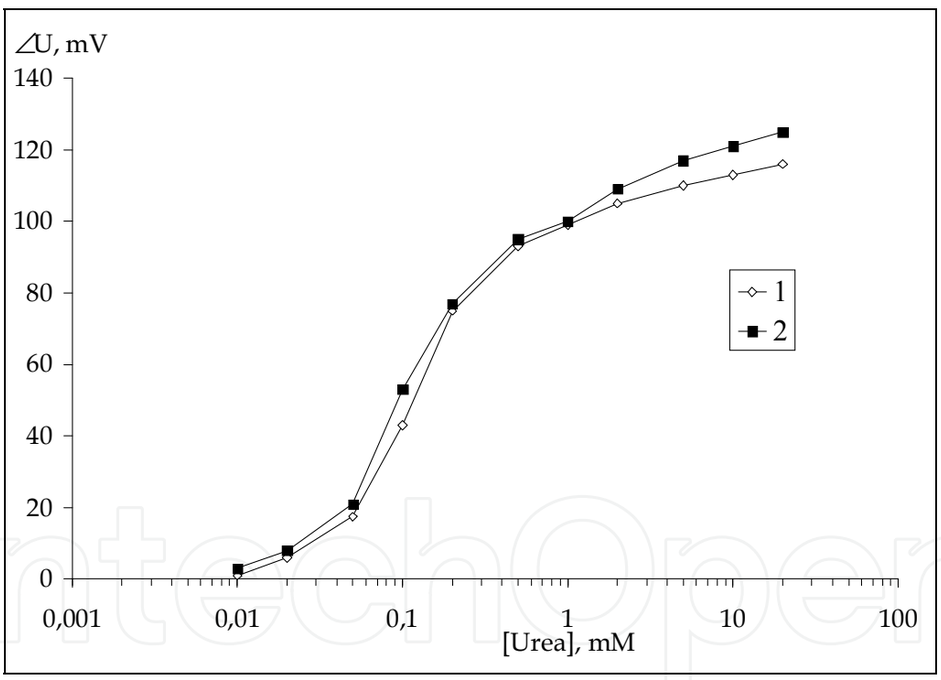


Fig. 16. Level of responses of the biosensors with membranes: fresh prepared (1), preserved (2) composition. Conditions of measurements: 1 mM of sodium phosphate buffer, pH 7,3.

Thus, an easy and fast method for immobilization of the enzyme urease on the surface of the IsFET gate is proposed. Based on the proposed bioactive membrane it was created biosensor for the express determination of urea in solution. Possibility of prolonged operation of the biosensor in real conditions was demonstrated. The conclusion about the possibility of recommendations developed photo polymerizable compositions for combining technologies of bioactive membrane production and manufacture of transducers, in particular, creation of IsFETs.

3. Conclusion

It was demonstrated that the proposed LPhPC is very suitable for the enzymatic biosensor creation. The process of the biological material immobilization on the surface of transducer can be done anyway phasic process and may be served as basis for technology of the biosensor production. Enzymes are long (up to 6 months) remaining active in staying as a part of the developed compositions, capable of photo polymerization and in the polymer membrane obtained from this composition. It was chosen the conditions (temperature, filtration of UV irradiation, the presence of competitive inhibitors) that increase the residual activity of immobilized enzymes. Extensively it was studied the properties of the developed electrochemical biosensors based on the IsFETs for the determination of glucose and urea as well it was show that they have the characteristics needed for use in laboratory, clinical, food and biotech practice.

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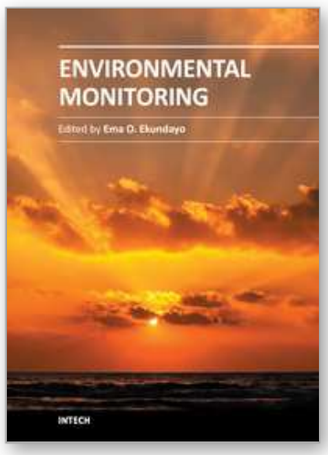
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