ISSN 0233-7657. Биополимеры и клетка. 1998. Т. 14. № 4

Biosensors based on conductometric detection

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The present paper is a self-review on the development of about 20 conductometric biosensors based on planar electrodes and containing different biological material (enzymes, cells, antibodies), bio-mimics or synthetic membranes, including imprinting polymers, as a sensitive element. Highly specific, sensitive, simple, fast and cheap determination of different analytes makes them promising for needs of medicine, biotechnology, environmental control, agriculture and food industry. Non-specific interference of background ions may be overcome by the differential mode of measurement, the usage of rather concentrated sample buffer and additional negatively or positively charged membranes, which decrease buffer capacity influence and extend a dynamic range of sensors response. For development of easy-to-use small conductometric immunosensors several approaches seem to be promising: i) the usage of polyaniline as electroconductive label for antibodies detection in competitive electroimmunoassay; ii) the elaboration of multilayer structures with phtalocyanine films; iii) the usage of acrylic copolymeric membranes. The advantages and disadvantages of conductometric biosensors created are discussed. For future commercialisation our effort are aimed to unite a thin-film technology with membranes deposition and to find the ways of membrane stabilisation, including bio-mimics creation, utilisation of bioaffinity polymeric membranes, imprinting polymers etc.

Introduction. The last decade has seen unprecedented interest in the development of analytical devices for the detection, quantification and monitoring of different biological and chemical compounds. The dynamic field of biosensors is covered by extensive number of reviews [1—5].

As a rule a biosensor is a device containing two functional parts: a bioselective membrane in direct contact with a physical transducer, which transforms the biochemical signal into the electrical or optical signal. The amplitude of this signal depends on the concentration of the analysed compound (analyte) in the sample. Biologically active materials used for construction of biosensor systems can be divided into two main groups: catalytic (enzymes, cells, tissues) and noncatalytic or affinity (antibodies, receptors, nucleic acids). Electrochemical (ampero-, potentio-, and conductometric), optical, calorimetric, and acoustic transducers are currently used in measuring systems.

The main efforts in biosensor development are focused on the exploration of the various combinations of biological components (or their synthetic mimics) with measuring principles, i. e. different transducers.

The conductometric sensors are based on the fact that many biochemical reactions in solution produce changes in the electrical resistance (reciprocal conductance) due to changes of ionic compounds. Conductance measurements usually involve the resistance determination of a sample solution between two parallel electrodes.

The biosensors based on conductometric principle seem to be the most advantageous in several aspects: i) thin-film electrodes are suitable for miniaturisation and large scale production using inexpensive technology; moreover, noble metals can be changed for cheaper ones, e. g. Ni; ii) they do not require reference electrode, have no light sensitivity, the driving voltage can be small to decrease power consumption substantially; iii) large spectrum of analytes of different nature can be determined on the basis of various reactions and mechanisms.

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The method for solution conductance monitoring was originally developed for determining chemical reaction rates and only recently it has been applied to reactions catalysed by enzymes [6—8], namely, urease [6, 9], proteases [8], oxidases [10], amidases and peptidases [11].

Conductometry is a unique method for direct assay of many enzymes and their substrates. According to Lawrence [7] five factors allow the application of conductometric methods for enzymatic analysis: 1) generation of ionic groups (e. g. amidases); 2) separation of unlike charges (e. g. decarboxylases); 3) proton migration (e. g. esterases); 4) changes in the degree of association of ionic groups resulting from chelation (e. g. kinases); and 5) changes in the sizes of charge-carrying groups (e. g. phosphatases). Factors 1 and 2 give rise to very large coefficients and make conductometry the assay method of choice.

Conductometric transducers for biosensing devices have been introduced by Watson et al. [12]. The device consisted of an oxidised silicon wafer with interdigitated gold electrodes pairs on one surface in a planar configuration.

The transducer was tested for urea detection in human serum samples [12] and for the enzymes penicillinase and acyl amilamidohydrolase activity study [13].

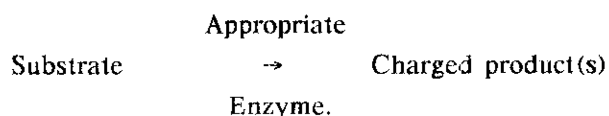
However, there was no sufficient progress in the development of the conductometric biosensors due to some disadvantages, mainly a very strong response dependence on a sample buffer capacity and a possible influence of non-specific ions.

Taking into consideration rather attractive features of conductometric principle and a simplicity of corresponding device, our efforts were aimed at the elaboration of different conductometric biosensors with improved working characteristics.

In this article we have reviewed our achievements in the development of conductometric biosensors based on planar electrodes and containing different biological material (enzymes, cells, antibodies), biomimics or synthetic membranes, including imprinting polymers, as a sensitive element.

Enzyme-based conductometric biosensors. The enzyme-based conductometric biosensors can be used for both direct and inhibitory analysis.

In case of direct analysis, the conductometric biosensors for determination of glucose, urea, acetylcholine chloride, butyrylcholine chloride and penicillin have been developed. The resulting conductivity changes are produced by enzymatically catalysed conversion of different substrates according to the general scheme of reaction:



The conductometric enzyme biosensors, employing the inhibitory analysis, have been created for organophosphorous pesticides and heavy metal ions determination. The scheme for pesticide detection is based on their ability to inhibit acetylcholinesterase and butyrylcholinesterase activity by phosphorylating the serine OH^- group in enzymes active sites. In the second case, immobilised urease can be inactivated by heavy metal ions through their direct interaction with the thiol group of the enzyme active site.

The assay protocol included measurement of the biosensor response to a fixed concentration of the specific substrate before and after the incubation of the biosensor for a definite time in a solution containing inhibitor.

The main analytical characteristics of created biosensors are presented in Table 1.

The biosensors demonstrated reproducible and stable response to the addition of substrates with a measurement time within 0.5—2 min. The influence of pH, buffer capacity and ionic strength of the samples on the biosensors response has been studied earlier and will be discussed further [14—16].

It is worth noting that the urease and cholinesterase-based conductometric biosensors may also serve as reliable tool for the estimation of the overall toxin level in liquid samples [17, 18].

However, all the enzyme sensors presented in Table 1 have been shown to possess strong dependence of their response on buffer capacity and ionic strength. The possibility to overcome this difficulty has been investigated on the glucose biosensor example [19]. The dynamic range of its response is very small and does not exceed 2 mM, the response value decreases 20-fold when the concentration of buffer is changed from 1 to 10 mM. Further increase of the buffer concentration up to 20—40 mM makes the measurement of glucose concentration practically impossible [16, 20]. The reason for the limited dynamic range of the glucose sensor is a lack of oxygen for biocatalytic oxidation of glucose [21]. The response dependence on buffer concentration may be explained by some kind of «carrier-mediated» transport of protons («facilitated diffusion») out of the enzyme membrane in the presence of mobile buffer species [19].

We have studied different additional membranes which may control diffusion of substrates and products of the biochemical reaction, thus optimising the

Table 1
Analytical characteristics of the enzyme biosensors created*

Substances	Dynamic range, M	Operational mode	Time of analysis, min	Stability	
				Operational	Storage
Glucose (GOD)	10^{-4}—$3\cdot10^{-3}$	Kinetic Steady-state	0.1—0.2 1—2	> 20 hs	90 day
Urea (urease)	10^{-4}—$5\cdot10^{-3}$	Kinetic Steady-state	0.1—0.2 1—3	> 20 hs	30 day
AcChCl (AChE)	10^{-4}—$1.2\cdot10^{-2}$	Kinetic Steady-state	0.1—0.2 1—2	> 10 hs	30 day
BuChCl (BuChE)	10^{-4}—10^{-2}	Kinetic Steady-state	0.1—0.2 1—2	> 10 hs	30 day
Penicillin (Penicillinase)	10^{-4}—$1.5\cdot10^{-3}$	Steady-state	1	> 20 hs	90 day
HSA (tripsin)	0.1—5 mg/ml	Steady-state	15	> 2 hs	30 day
		Inhibitory analysis			
Hg^{2+}	10^{-6}—$5\cdot10^{-5}$	Kinetic	20**	Disposable	30 day
Cu^{2+}	$2\cdot10^{-6}$—10^{-4}	Kinetic	20**	Disposable	30 day
Cd^{2+}	$5\cdot10^{-6}$—$2\cdot10^{-4}$	Kinetic	20**	Disposable	30 day
Co^{2+}	10^{-5}—$5\cdot10^{-4}$	Kinetic	20**	Disposable	30 day
Pb^{2+}	$2\cdot10^{-5}$—$5\cdot10^{-3}$	Kinetic	20**	Disposable	30 day
Sr^{2+}	10^{-4}—$5\cdot10^{-3}$	Kinetic	20**	Disposable	30 day
DFP	$5\cdot10^{-11}$—10^{-7}	Steady-state	15**	> 2 hs	30 day
Trichlorfon	$5\cdot10^{-7}$—10^{-5}	Steady-state	15**	> 2 hs	30 day
Paraoxon-ethyl	10^{-8}—10^{-4}	Steady-state	15**	> 2 hs	30 day
Paraoxon-methyl	$5\cdot10^{-7}$—10^{-4}	Steady-state	15**	> 2 hs	30 day

*Without additional charged membranes; **measurement time includes preincubation with inhibitor.

sensor operation in order to satisfy practical requirements [19—23].

It is shown in Fig. 1, that the deposition of a negatively charged Nafion membrane (thickness about 3.5 μm) on the top of the enzymatic film extends the dynamic range of the sensor up to a concentration of glucose of 4 mM and makes the sensor response practically insensitive to an increase of buffer concentration from 1 to 10 mM. In the following experiments we tested the dependence of the sensor response on the Nafion membrane thickness (Fig. 2). With an increase of the membrane thickness up to 15 μm the value of the sensor response increases, and the dynamic range extends up to 10 mM concentration of glucose.

The results obtained can be interpreted in terms of permselective properties of the additional Nafion membrane formed. The presence of negatively charged groups of the Nafion membrane blocks so called «carrier-mediated» transport of protons through the additional membrane, thus eliminating the effect of mobile buffer species on the amplitude of the output signal of biosensor. The additional Nafion membrane also limits the diffusion of glucose through the

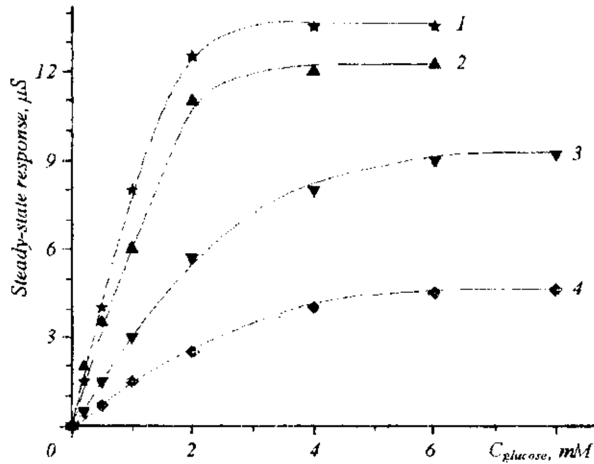


Fig. 1. Calibration curves for the steady-state response of the glucose sensor with additional Nafion membrane in 1 mM (*1*), 10 mM (*2*), 20 mM (*3*) and 40 mM (*4*) phosphate buffer, pH 7.5

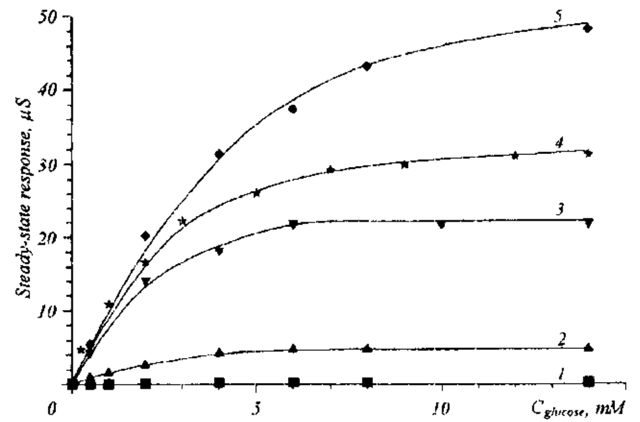


Fig. 2. Calibration curves for steady-state response of the glucose sensor without (*1*) and with one (*2*), two (*3*), three (*4*) and four (*5*) layers of the additional Nafion membrane in 40 mM phosphate buffer, pH 7.5

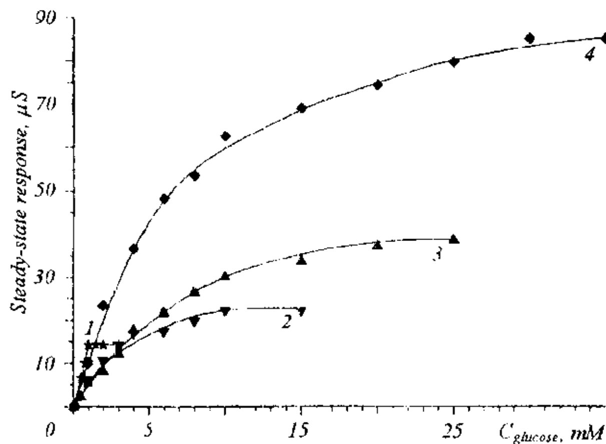


Fig. 3. Calibration curves for steady-state response of the glucose sensor without (*1*) and with one (*2*), two (*3*) and three (*4*) layers of the additional 0.5 % poly(4-vinylpyridine-co-styrene) membrane in 5 mM phosphate buffer, pH 7.5

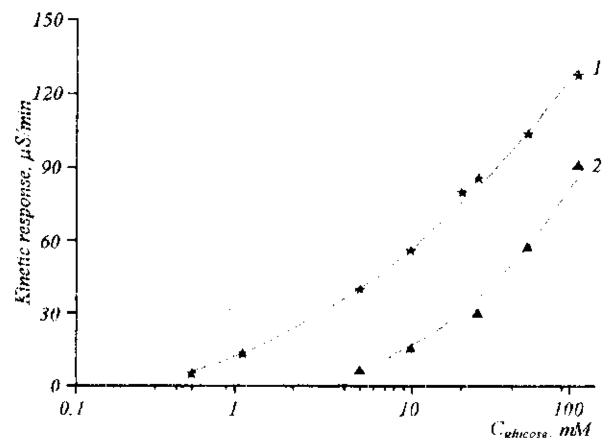


Fig. 4. Calibration curves for cell-based conductometric biosensor. Measurements were performed in distilled water (*1*) or polymix buffer (*2*) (pH 7.0, buffer capacity 2 mM/pH; cell concentration in biomembrane 20 mg/ml; $CaCl_2$ concentration 10 mM)

membrane which results in an extension of the sensor dynamic range.

As for the positively charged membrane, Fig. 3 shows that 0.5 % poly(4-vinylpyridine-co-styrene) membranes of different thickness extend the dynamic range of the sensor up to a concentration of glucose of 30 mM.

This phenomenon may be explained by the limitation of proton diffusion out of the membrane. The decrease of response dependence on buffer

capacity due to this membrane depends on the buffer type.

Cell-based conductometric biosensors. Yeast cell-based conductometric biosensor with planar interdigitated gold electrodes has been performed for the quantification of ethanol in alcoholic beverages [24]. When ethanol is added to the tested solution, alcohol molecules penetrate through cell membrane and are oxidised in two steps by methylotrophic yeast *Candida boidinii* 706 (wild type). First of all, alcohol dehydrogenase (type II) catalyses the formation of acetaldehyde in the oxidative utilisation of ethanol. Then, acetaldehyde is converted to acetic acid by acetaldehyde dehydrogenase. The resulting conductivity changes due to the specific acidic metabolite secretion out of the membrane are registered by the sensor system.

Fig. 4 shows the calibration curves for ethanol biosensor which are linear in the range of analyte concentration from 5 mM to 100 mM.

The microbial sensor can be used with the same biological membrane several times after 30 min interval needed for washing and system stabilising. Relative standard deviation of the measurements is 10—12 % for 15 repeated assays. A decrease of the sensor output in slightly buffered sample solutions has been found. The biosensor signal is stable for 12 days, when the system is operated 2 hours daily and then stored at 4 °C.

The results of ethanol determination in different diluted alcoholic beverages by the cell-biosensor created as compared to those obtained by method of gas chromatography are presented in Fig. 5. It is shown, that each value coincided relatively well with that determined by the reference method with a correlation coefficient 0.9988.

Conductometric immunosensors. Despite the excellent sensitivity of ELISA and fluorescent immunoassay, their application is limited sometimes by short lifetime of labels, necessity of complex and expensive equipment, relatively long time of analysis. So alternative procedures may be of special interest for express immunological testing.

For the development of easy-to-use small conductometric immunosensors several approaches seem to be promising: i) the usage of polyaniline as electroconductive label for antibodies detection in competitive electroimmunoassay; ii) the elaboration of multilayer structures with phthalocyanine films; iii) the usage of acrylic copolymeric membranes.

In the first case the conductometric scheme of a sensor is the same as described above. A non-competitive mode of antibodies detection has been employed with the usage of polyaniline (PA) as an electrically conductive label. Various water-soluble forms of PA with different molecular weights and oxidation levels have been compared. The maximum specific response has been observed for the PA-

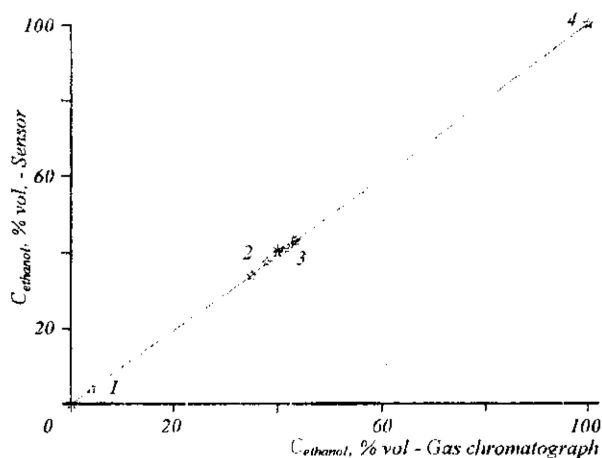


Fig. 5. Results of ethanol determination in different alcoholic beverages (beer (*1*), vodka (*2*), cognac (*3*) and spirits (*4*))

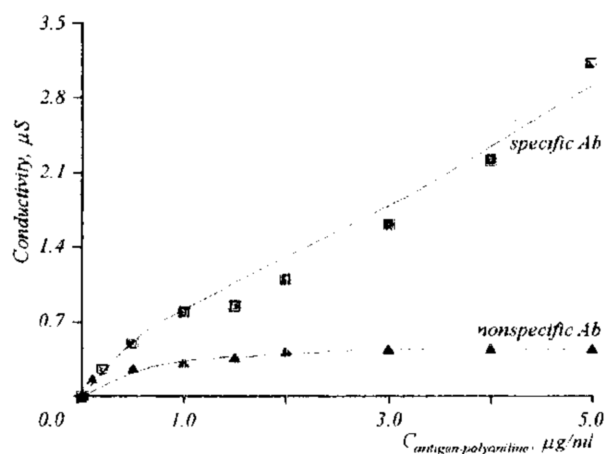


Fig. 6. Dependence of the sensor response with electroactive polyaniline label on antigen-polyaniline conjugate concentration

fraction of 4.5 kDa, synthesised in the presence of 0.2 mM ammonium peroxidisulfate. The sensor response for the specific Ab-PA conjugate has been revealed to be three times larger than for non-specific one. Minimum quantity of labelled antibodies easily detected is 50 ng/ml (Fig. 6). At the competitive mode of antibodies detection the sensor sensitivity is lower, which needs further improvement.

The second approach to conductometric immunosensor creation utilises iodine-sensitive phthalocyanine thin-films [25]. Excellent sensitivity of the tetra-tert-butyl CuPc (ttb-CuPc) to free iodine is used in an aqueous medium to detect the peroxidase reaction. To minimise interfering effect of aqueous electrolytes Au/Cr interdigitated planar electrodes bearing ttb-CuPc thin-films were overcoated with a hydrophobic gas-permeable membrane. After optimising the sensor working parameters the detection of peroxidase-labelled antibodies in the range of 0.1—2 μg/ml has been demonstrated. Therefore, for the first time, the chemoresistor based on organic semiconductor, phthalocyanine, has been used as a conductometric transducer for biosensors operating in aqueous media.

However, phthalocyanines main shortcomings are evident: low conductivity and rather slow response, and this stimulates a search for alternative chemoresistive compounds exhibiting good chemical sensitivity, high conductivity and faster response.

A new way to detect different antibodies or antigens is based on the usage of copolymers of acrylonitrile with acrylic acid [26, 27]. The films cased from such polymer are stable and flexible. Electroconductivity (resistance) of such membranes is determined by two factors: properties of the polymer surface and material porosity. Immunochemical reactions between one of the immunocomponents being immobilised within the porous membrane and the other in analysed sample causes changes in the membrane charge distribution and porous structure which results in alteration of the membrane resistance and can be used for development of immunosensors. The response of such sensor system depends on the polymer composition and conditions of membrane formation.

It has been shown that during Ab-Ag binding the membrane conductivity increases with the increase of analysed Ag concentration. However, the porous structure must be optimised to ensure: i) maximum binding of free antigen in solution by biomembrane with immobilised antibody; ii) maximum influence of the immunological reaction on the ion transfer, responsible for the membrane electroconductivity; iii) occurring of immunological reaction mainly inside of

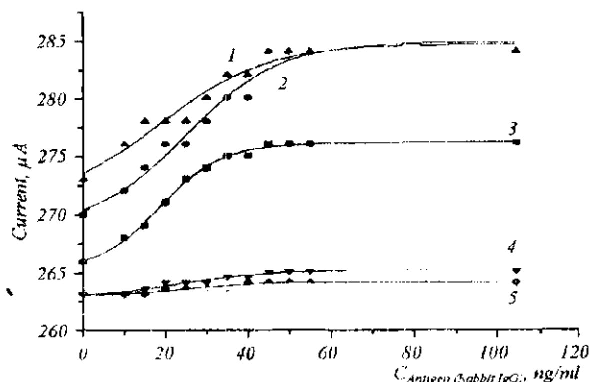


Fig. 7. Calibration curves for «bioaffinity» membranes based on copolymers of acrylonitril with acrylic acid (*1* — 18 % polymer, membrane 0.3 mm; *2* — 18 % polymer, membrane 0.2 mm; *3* — 16 % polymer, membrane 0.3 mm; *4* — BSA; *5* — non-specific antigen)

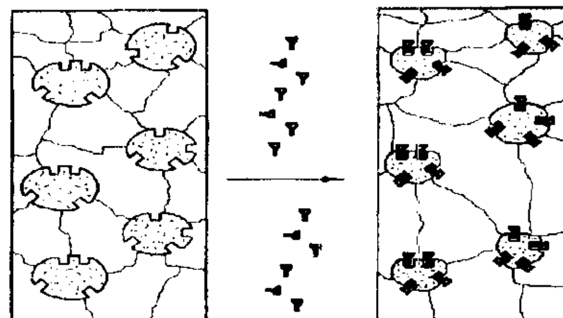


Fig. 8. Possible changes in imprinted polymer structure under reaction with template molecules inducing changes in conductance

micropores since the rate and value of immunosensor response depends on this factor significantly.

Fig. 7 demonstrates that membranes prepared from 18 % polymer and characterised by more ordered structure, especially almost optimised porosity, possess the highest sensitivity for specific antigen.

Thus, the electric method for detecting immunochemical reaction in solution has been developed. The advantages of the immunosensor described are rather high sensitivity (about 5 ng/ml of antigens can be detected) and fast response (1—2 minutes) at satisfactory specificity.

Imprinting polymers in sensor design. Unfortunately, the stability of antibodies, enzymes, cells and other biological components often appeared limi-

ted for the creation of commercialised devices for a number of analytes. That is why the development of simple, effective and technological methods for creation of highly-selective analytical systems without unstable biological material is of great importance.

Stable synthetic mimics of receptor (or antibody) and enzyme binding sites can be designed by imprinting technique, first reported by Wulff [5]. In general, cross-linked polymers are formed around a molecule that acts as a template, and this template is then removed. An imprint containing reactive groups with binding capability remains in the polymer. The structure of well-defined cavities and arrangement of functional groups are predetermined by the chemical nature of the template. Subsequent recognition occurs via a combination of reversible binding and shape complementary (Fig. 8).

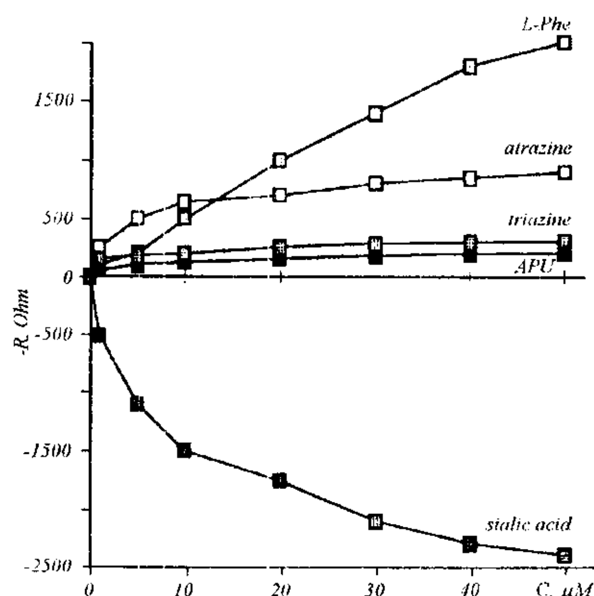


Fig. 9. Dependence of the response of imprinted polymer-based sensors on template concentration

Using molecular imprinting approach new types of polymeric membranes containing molecular recognition sites for L-phenylalanine (L-Phe), 6-amino-1-propyluracil (APU), atrazine and sialic acid have been prepared [28]. The membrane synthesis includes radical polymerisation of ethylene glycol dimethacrylate and functional monomer in the presence of template. Several substances—diethyl aminoethyl methacrylate, methacrylic acid, allylamine, or vinylphenylboronic acid have been tested as functional monomers, able to form covalent, ionic or hydrogen bonds with corresponding templates. After splitting off the template molecules, these polymers have been used as materials for conductometric sensors, specific for a corresponding template (Fig. 9). The sensors created detect the analytes in solution in the range of 1—50 μM concentrations (Table 2).

Besides high sensitivity, analytical devices containing imprinted polymers are expected to be extremely stable and operate in such conditions, where usual biosensors are ineligible, which is especially important for health and environmental control and protection.

Table 2
Analytical characteristics of the matrix biosensors created

Substances	Dynamic range, M	Operational mode	Time of analysis, min	Stability	
				Operational	Storage
Atrazine**	$5\cdot10^{-6}$—$5\cdot10^{-5}$	Steady-state	40	3 month*	2 years
Triazine	10^{-5}—10^{-3}	Steady-state	40	3 month*	2 years
Cholesterol	10^{-6}—$5\cdot10^{-4}$	Steady-state	40	3 month*	2 years
L-Phenylalanine	10^{-6}—$5\cdot10^{-3}$	Steady-state	40	3 month*	2 years
AMP	$5\cdot10^{-6}$—10^{-3}	Steady-state	40	3 month*	2 years
Sialic acid	$5\cdot10^{-7}$—$5\cdot10^{-5}$	Steady-state	40	3 month*	6 months
6-Amino-1-propyluracil**	$5\cdot10^{-6}$—10^{-4}	Steady-state	40	3 month*	2 years

*Stability with regeneration; **the sensors were developed by Dr. Piletsky under supervision of prof. I. Karube (Research Center for Advanced Science and Technology, University of Tokyo, Japan).

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Біосенсори на основі кондуктометрії

Резюме

Огляд присвячено аналізу власних робіт з розробки близько 20 кондуктометричних біосенсорів на основі планарних електродів та різноманітного біологічного матеріалу (ферменти, клітини, антитіла), синтетичних мембран як чутливих елементів. Висока селективність, чутливість, низька ціна, простота та експресність визначення різноманітних речовин роблять біосенсори необхідними для потреб медицини, біотехнології, екології, сільського господарства та харчової промисловості. При аналізі реальних зразків неспецифічний вплив фонових електролітів можна суттєво зменшити завдяки використанню диференційного режиму вимірювань, більш концентрованих буферних розчинів, а також додаткових негативно чи позитивно заряджених мембран, які запобігають впливові буферної ємності та іонної сили розчинів і розширюють динамічний діапазон роботи сенсорів. Для створення мініатюрних імуносенсорів було запропоновано такі підходи: а) використання поліаніліну як електропровідної мітки при визначенні антитіл у конкурентному імуноаналізі: б) створення багатошарових структур з плівками фталоціаніну; в) використання акрилових сополімерних мембран. Обговорено переваги та недоліки розроблених кондуктометричних біосенсорів. Подальша комерціалізація таких приладів пов'язана з пошуком шляхів стабілізації чутливих мембран та суміщення тонкоплівкових технологій з нанесенням мембран у єдиному технологічному циклі.

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Биосенсоры на основе кондуктометрии

Резюме

Обзор посвящен анализу собственных работ по разработке около 20 кондуктометрических биосенсоров на основе планарных электродов и различного биологического материала (ферменты, клетки, антитела) и синтетических мембран в качестве чувствительных элементов. Высокая селективность, чувствительность, дешевизна, простота и быстрота определения различных веществ делают биосенсоры необходимыми в медицине, биотехнологии, экологии, сельском хозяйстве и пищевой промышленности. При анализе реальных образцов неспецифическое влияние фоновых электролитов можно устранить благодаря использованию дифференциального режима измерений, более концентрированных буферных растворов, а также дополнительных отрицательно или положительно заряженных мембран, уменьшающих влияние буферной емкости и ионной силы растворов и расширяющих динамический диапазон работы сенсоров. Для создания миниатюрных иммуносенсоров предложены следующие подходы: а) использование полианилина как электропроводящей метки при определении антител в конкурентном иммуноанализе; б) создание многослойных структур с пленками на основе фталоцианина; в) использование акриловых сополимерных мембран. Обсуждены преимущества и недостатки разработанных кондуктометрических биосенсоров. Дальнейшая коммерциализация таких приборов связана с поиском путей стабилизации чувствительных мембран и совмещения тонкопленочной технологии с нанесением мембран в едином технологическом цикле.

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Received 20.01.98