

ISSN 2518-1629 (Online),  
ISSN 2224-5308 (Print)

ҚАЗАҚСТАН РЕСПУБЛИКАСЫ  
ҰЛТТЫҚ ҒЫЛЫМ АКАДЕМИЯСЫНЫҢ  
Өсімдіктердің биологиясы және биотехнологиясы институтының

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## ИЗВЕСТИЯ

НАЦИОНАЛЬНОЙ АКАДЕМИИ НАУК  
РЕСПУБЛИКИ КАЗАХСТАН  
Института биологии и биотехнологии растений

## NEWS

OF THE NATIONAL ACADEMY OF SCIENCES  
OF THE REPUBLIC OF KAZAKHSTAN  
of the Institute of Plant Biology and Biotechnology

**БИОЛОГИЯ ЖӘНЕ МЕДИЦИНА  
СЕРИЯСЫ**



**СЕРИЯ**

**БИОЛОГИЧЕСКАЯ И МЕДИЦИНСКАЯ**



**SERIES**

**OF BIOLOGICAL AND MEDICAL**

**6 (330)**

**ҚАРАША – ЖЕЛТОҚСАН 2018 ж.  
НОЯБРЬ – ДЕКАБРЬ 2018 г.  
NOVEMBER – DECEMBER 2018**

**1963 ЖЫЛДЫҢ ҚАҢТАР АЙЫНАН ШЫҒА БАСТАҒАН  
ИЗДАЕТСЯ С ЯНВАРЯ 1963 ГОДА  
PUBLISHED SINCE JANUARY 1963**

**ЖЫЛЫНА 6 РЕТ ШЫҒАДЫ  
ВЫХОДИТ 6 РАЗ В ГОД  
PUBLISHED 6 TIMES A YEAR**

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«ҚР ҰҒА Хабарлары. Биология және медициналық сериясы».

**ISSN 2518-1629 (Online),**

**ISSN 2224-5308 (Print)**

Меншіктенуші: «Қазақстан Республикасының Ұлттық ғылым академиясы» РҚБ (Алматы қ.)

Қазақстан республикасының Мәдениет пен ақпарат министрлігінің Ақпарат және мұрағат комитетінде  
01.06.2006 ж. берілген №5546-Ж мерзімдік басылым тіркеуіне қойылу туралы куәлік

Мерзімділігі: жылына 6 рет.

Тиражы: 300 дана.

Редакцияның мекенжайы: 050010, Алматы қ., Шевченко көш., 28, 219 бөл., 220, тел.: 272-13-19, 272-13-18,  
[www.nauka-nanrk.kz/biological-medical.kz](http://www.nauka-nanrk.kz/biological-medical.kz)

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Типографияның мекенжайы: «Аруна» ЖК, Алматы қ., Муратбаева көш., 75.

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**«Известия НАН РК. Серия биологическая и медицинская».**

**ISSN 2518-1629 (Online),**

**ISSN 2224-5308 (Print)**

Собственник: РОО «Национальная академия наук Республики Казахстан» (г. Алматы)

Свидетельство о постановке на учет периодического печатного издания в Комитете информации и архивов  
Министерства культуры и информации Республики Казахстан **№5546-Ж**, выданное 01.06.2006 г.

Периодичность: 6 раз в год

Тираж: 300 экземпляров

Адрес редакции: 050010, г. Алматы, ул. Шевченко, 28, ком. 219, 220, тел. 272-13-19, 272-13-18,  
[www.nauka-nanrk.kz](http://www.nauka-nanrk.kz) / [biological-medical.kz](http://biological-medical.kz)

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**News of the National Academy of Sciences of the Republic of Kazakhstan. Series of biology and medicine.**

**ISSN 2518-1629 (Online),**

**ISSN 2224-5308 (Print)**

Owner: RPA "National Academy of Sciences of the Republic of Kazakhstan" (Almaty)

The certificate of registration of a periodic printed publication in the Committee of information and archives of the Ministry of culture and information of the Republic of Kazakhstan N 5546-Ж, issued 01.06.2006

Periodicity: 6 times a year

Circulation: 300 copies

Editorial address: 28, Shevchenko str., of. 219, 220, Almaty, 050010, tel. 272-13-19, 272-13-18,  
<http://nauka-nanrk.kz> / [biological-medical.kz](http://biological-medical.kz)

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Address of printing house: ST "Aruna", 75, Muratbayev str, Almaty

## NEWS

OF THE NATIONAL ACADEMY OF SCIENCES OF THE REPUBLIC OF KAZAKHSTAN

SERIES OF BIOLOGICAL AND MEDICAL

ISSN 2224-5308

Volume 6, Number 330 (2018), 5 – 12

<https://doi.org/10.32014/2018.2518-1629.11>

UDC 577.3 620.3 578.08.002.5

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**DEVELOPMENT OF A BIOSENSOR OF UREA  
WITH THE APPLICATION OF POLYMER TECHNOLOGIES  
FOR BLOOD AND URINE ANALYSIS**

**Abstract.** Based on polymeric nanotechnologies, enzyme sensors and microreactors have been developed in the way, that they can determine urea in liquids. The technology of manufacturing an enzymatic biosensor does not differ significantly from the known technology of manufacturing microcapsules with an enzyme by the layer-by-layer method. This allows us, when constructing a biosensor, to use the information obtained on encapsulated enzymes by other authors. It is shown that urea biosensor is able to work for a long time (up to 2 months) without significant loss of enzyme activity. Polymer technology for manufacturing sensors is less laborious and expensive compared to other similar technologies. We propose to develop biosensor devices – urea analyzers with polymer enzyme chips for express diagnostics of biological fluids (blood, urine). One of the significant results of this work from our point of view is two factors. The first factor is the optimization of the conditions for the production of a functionally active enzyme immobilized in a polyelectrolyte coating, when the enzyme after the immobilization procedure shows an activity comparable to that of a freshly prepared free enzyme. Such a result will allow reducing the cost of enzymes when creating a sensitive layer of the developed urea analyzer. And the second factor is that the polymer coating with the enzyme is able to work not only as an enzyme electrode, but also as an enzyme microreactor, without decreasing the rate of signal registration after passing the catalytic urease-urea reaction.

**Keywords:** enzyme biosensors, polymeric nanomaterial, portable analyzer, microreactor, microcapsules, urea.

**Introduction.** The volume of laboratory research worldwide is steadily increasing and reaches 45 billion analyzes per year, and in industrialized countries the number of analyzes per person reaches 40-60 per year. Universal biochemical analyzers analyze any biological fluids (substrates, enzymes, lipids, drugs, hormones, proteins, electrolytes, drugs). They are produced by about 60 companies, the main producers are Abbott (USA), ABC1 (Austria), Koné (Finland), Nova (USA), Corning (England), Beckmann (USA), "Radiometer" (Denmark). Ready-made sets of reagents are in great demand. Their market is about 27 billion dollars in the world market of laboratory instruments in 6 billion dollars.

Spectroscopic analyzers are used for biochemical studies (determination of organic and inorganic chemicals, such as potassium, sodium, calcium, magnesium, lithium, chlorine, substrates, metabolites, enzymes of biochemical processes in blood and other human biological fluids). Universal biochemical analyzers with the help of which an analysis of any biological fluids for the content of various components are recognized as promising. However, at the present time there are no portable devices of this class. The development of portable devices for the analysis of biological fluids is an urgent task of modern medical diagnostics. Of particular interest among portable analyzers of various substances undoubtedly represent analyzers based on biosensors. Any biosensor consists of two functional elements: a biosensor containing a bioselective material, and a physical converter that transforms any generated signal (ion concentration, mass, color, etc.) into an electrical signal. In the role of bioselecting material are all types of biological structures - enzymes, antibodies, receptors, nucleic acids and even living cells. In biosensors are used a variety of physical converters: amperometric, conductometric, optical, luminescent, fluorescent, acoustic, gravitational, etc.

The development of biosensors is an extremely time-consuming process. The most important stage in the development of enzyme sensors is proper immobilization of enzymes on solid supports (substrates). We have developed a method for immobilizing enzymes using polymer technologies, in which the immobilized enzyme is in a functionally active state [1-3]. Immobilization of enzymes was carried out in a biosensor sensitive coating, which is a combination of nanometer polyelectrolyte layers and micro-encapsulated enzymes placed between these layers (figure 1).

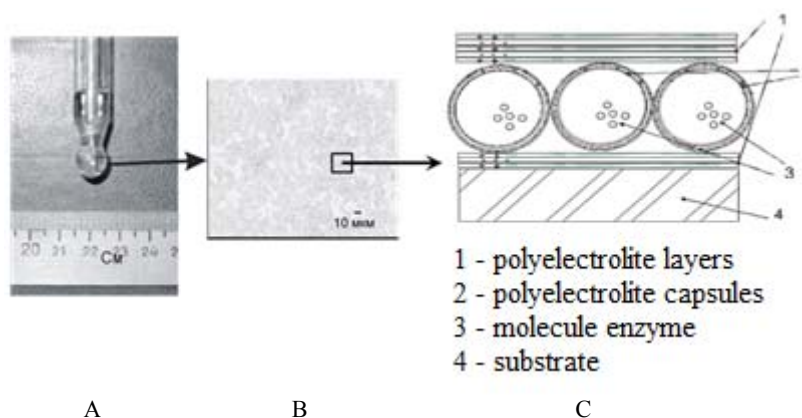


Figure 1 – Enzyme electrode with sensitive biosensor coating:  
A – is a glass pH electrode with a sensitive coating containing the enzyme urease;  
B – image of a polyelectrolyte coating with microcells in a light microscope;  
C – is a schematic representation of a sensitive coating with an enzyme

As it was shown in these works, enzymes in microcells of a polymeric material are reliably protected from aggressive influences of environment (microbes, proteases, etc.); able to detect substrates for a long time (up to 3 weeks in storage at room temperature). This work continues to improve the characteristics of the developed urea biosensor.

**Materials and methods.** For the production of enzymatic biosensors and enzyme micro-reactors, lyophilized urease (EC 3.5.1.5) was used from the *Canavalia ensiformis* beans of Sigma and Fluka, an urease solution from the Urea KT(200) kit, (Deacon-DS) with an activity of 253000 U/l., Urea extra clean (Reachim), MES buffers (Sigma), Tris-HCl (Sigma). Salts of  $\text{CaCl}_2$ ,  $\text{Na}_2\text{CO}_3$ , NaCl and KCl had a gradation of chemically pure or pure for analysis. Ethylene glycoltetraacetic (EGTA) and ethylenediaminetetraacetic (EDTA) acid (both Sigma-Aldrich, USA). To form films and shells of microcapsules, domain enzymes, polyelectrolytes were used: polyethyleneimine (PEI) weight 600000-1000000, polystyrene sulfonate (PSS), polyallylamine hydrochloride (PAAH), (all - Aldrich) with a mass of 60000-70000. The test substances were used as solutions in 0.33 M NaCl. All salt solutions were prepared on deionized water obtained by purifying distilled water with Arium 611-UF (Sartorius). The conductivity of the water was 1  $\mu\text{S}/\text{cm}$ .

The following instruments were used in the work: spectrophotometer Bekman UV/Vis DU 520 (USA), Nikon eclipse E200 microscope, 4-channel potenti-microamperometric analog-digital amplifier "Record-4usb" with computer connection (development of IBK RAS), pH- meter Bekkman F 690 pH / Temp/mV/ISE Meter (USA), Axiovert 200 microscope, photometer (model 680 BIO-RAD, USA), Vortex (shaking and mixing device), ultrasonic bath, magnetic stirrer, table centrifuge, semi-automatic micro-pipette for 2-20  $\mu\text{l}$ , 20-200  $\mu\text{l}$ , 200-1000  $\mu\text{l}$ , 5000  $\mu\text{l}$ , chamber Goryaev.

*Preparation of enzyme-containing calcium carbonate crustal particles.* Composite microspherulites  $\text{CaCO}_3$ - protein were used as core microparticles for the preparation of polyelectrolyte capsules.

$\text{CaCO}_3$  microspherulites were obtained by the ion exchange reaction when mixing solutions of calcium chloride and carbonate in the presence of protein (enzyme) – by biomineralization [4-7].

*Preparation of enzyme-containing polyelectrolyte microcapsules.* Polyelectrolyte microcapsules with urease were produced by the method of alternate layer-by-layer adsorption with the application of polystyrene sulfonate (PSS) and polyallylamine hydrochloride (PAAH) molecules to composite calcium-carbonate spherulites containing urease as described in [4-6, 8].

Alternate layering of oppositely charged macromolecules of polyelectrolytes on colloidal particles was carried out three to five times, obtaining three/five shells with the architecture of PAAH/(PSS/PAAH)<sub>n</sub> and PSS/(PAGE/PSS)<sub>n</sub> where n=1,2. The procedure for the formation of microcapsules was carried out at room temperature (15-25°C). Microcapsule size and sphericity of calcium carbonate particles were monitored with a Nikon eclipse E200 light microscope. The removal of calcium carbonate particles from the microcapsules was carried out while maintaining the solution with microcapsules in dialysis bags for 3 hours to 12-15 hours in 25 mM EGTA or EDTA at a temperature of 4°C or 20°C with basic alkalization (pH 7.2-7.5). The number of capsules in the solution was counted using a camera Goryaev.

*Potentiometric method for determination of urea concentration with a standard pH electrode.* Using the technique described in [1, 2], a potentiometric polymer biosensor of urea was prepared on the basis of a modified glass pH electrode (figure 1A). Measurements of the hydrogen ion concentration in the test solution were carried out using a four-channel ADC – "Record 4usb". The solution was stirred with a magnetic stirrer and maintained at 25 ± 1 °C with a U-1 thermostat (Germany). Then, the enzyme preparation was added thereto in the required quantities or a modified pH electrode was introduced. The alkaline pH shift recorded (in mV) was saturated for 20-30 seconds.

**Results and discussion.** For the first time, the possibility of measuring the urea concentration by a modified glass pH electrode on which an ultrathin sensitive polymer coating with urease was deposited was demonstrated by us in [1, 2]. The following properties of the polymer coating provided this possibility: good permeability of polyelectrolyte multilayers for the substrate (urea) and its decomposition products by urease; impermeability of these layers for the enzyme; preservation of the enzyme in the cells of the coating, high activity for a sufficiently long time; as well as significant alkalization of the medium during the decomposition of urea to carbon dioxide and ammonia. Improving the characteristics and properties of the polymer sensitive coating of the urea sensor is associated with an increase in the initial activity of the immobilized enzyme, an increase in the duration of the sensor operation, and the ability to measure urea in biological fluids. As was shown in [9], we managed to achieve a sufficiently high activity of the immobilized enzyme, which amounted to 40-50% of the activity of the free freshly prepared enzyme.

In this paper, data are presented on the continuation of studies related to an increase in the initial activity of the urease sensor. Figure 2 shows the data on the dependence of the response of the glass pH electrode on urea concentration in the measuring cell for the free (line 1) and encapsulated (line 2) enzyme.

It can be seen from the figure that the activity of the encapsulated enzyme is comparable to the activity of a free freshly prepared enzyme and was about 75% of its activity. In the encapsulation process, the enzymes are partially damaged, and in the first studies on capsules with the enzyme, a high initial

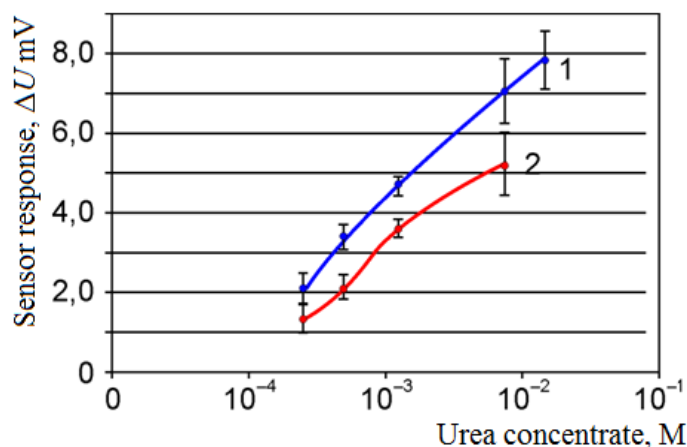


Figure 2 – Dependence of the response of the glass pH electrode on the urea concentration (0.5 μg enzyme concentration was determined by the Bradford method): 1 - free enzyme (urease); 2 - encapsulated enzyme contained in microcapsules with the architecture of the PSS-PAAG-PSS envelope. Study medium: 1 mM Tris-HCl, 1 mM MES, 100 mM NaCl, initial pH 5.3.

activity of the encapsulated enzyme was not achieved. Usually, the activity of encapsulated enzymes decreased by a factor of 6-7 [1, 6, 10-15]. Close results to our experimental data presented in this study on encapsulated urease were obtained in [16-19]. Authors, using the enzyme dextranase, obtained encapsulated enzymes with a catalytic activity equal to 80% of the activity of the free enzyme (the capsules were formed from calcium alginate with the inclusion of silica).

Since unmodified glass pH electrodes were used for measurements in cells with a free and encapsulated enzyme, we tried to compare pH measurements during the passage of the urease-urea catalytic reaction using a modified by our method an electrode and an unmodified electrode that were simultaneously placed in a measuring cell. In this case, the decomposition reaction of urea passed in the biosensitive layer of the modified electrode (figure 3).

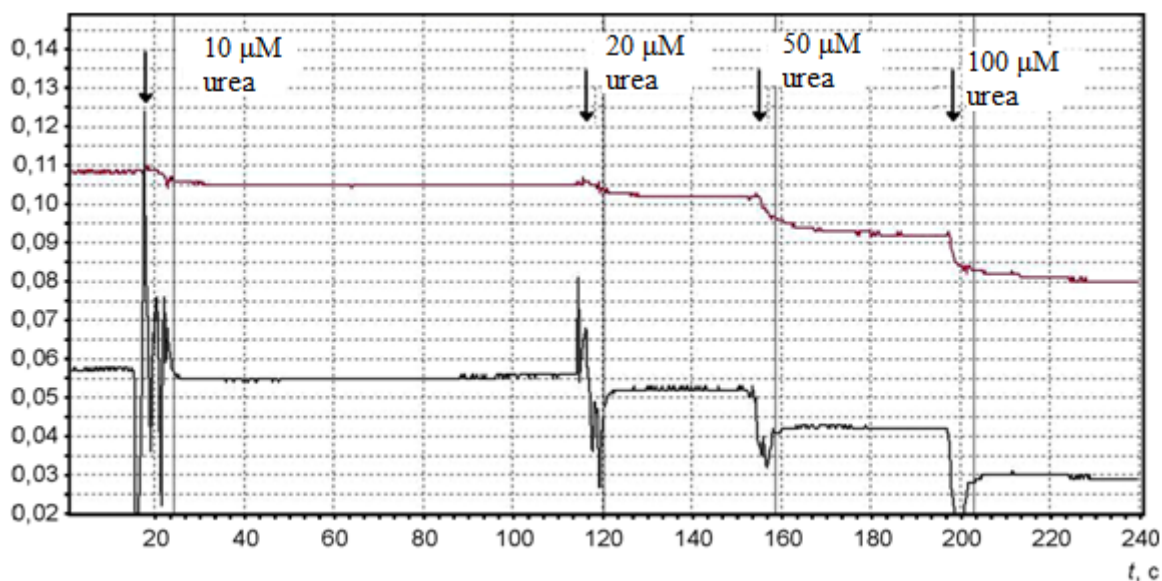


Figure 3 – Diagram of the experiment for measuring urea concentration with unmodified (upper curve) and modified pH electrodes.

A sensitive coating with urease is deposited on the ball of the lower electrode. Microcells of sensitive coating with the architecture of the shell of PAAG-PSS-PAAG.

Study medium: 1 mM MES, 100 mM NaCl, initial pH 6.0

Since unmodified glass pH electrodes were used for measurements in cells with a free and encapsulated enzyme, we tried to compare pH measurements during the passage of the urease-urea catalytic reaction using a modified by our method an electrode and an unmodified electrode that were simultaneously placed in a measuring cell. In this case, the decomposition reaction of urea passed in the biosensitive layer of the modified electrode (figure 3).

It can be seen from the experimental diagram that the response time after the catalytic reaction of the enzyme-substrate with the help of the modified and unmodified electrodes is practically the same. This is due to the fact that the substrate – urea and the decay products of the urease-urea catalytic reaction – carbon dioxide and ammonia easily penetrate through the nanometer polyelectrolyte shell that separates urease from the external solution. Such experimental results allowed us to create not only enzyme electrodes, but also enzyme microreactors (when the recording electrode is separated from the sensitive layer).

As a microreactor, plastic and glass cuvettes with a polyelectrolyte coating were applied, the same as for a ball of a modified pH electrode. This coating, which is a multilayer film, between layers of which was a layer of polyelectrolyte capsules with a diameter of about 2-5 microns filled with urease molecules, was applied to one of the walls of the cuvette. The presence of several, not less than five polyelectrolyte layers separating enzymes from the external environment, prevented the latter from inactivation, for example, by foreign enzymes or microbes. One of the features of the coating was that the total thickness of the polymer layers was less than 2% of the inner cell diameter in it.

Figure 4 presents data on the catalytic activity of the urease microreactor.



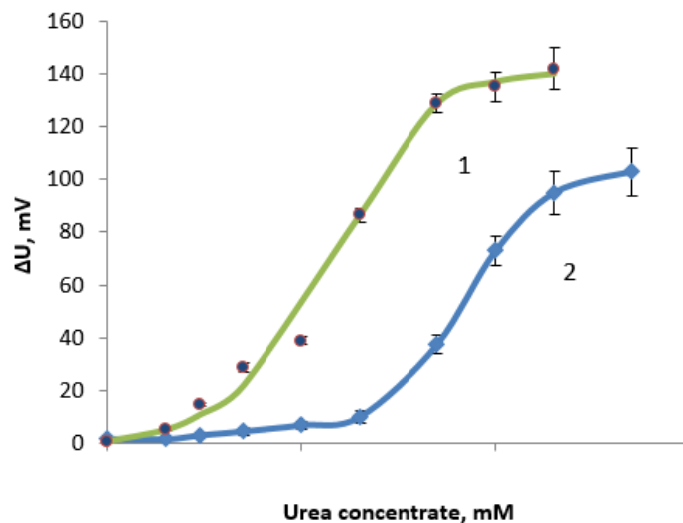


Figure 4 – Dependence of the response of the glass pH electrode on urea concentration (enzyme concentration 3  $\mu\text{g}$  was determined by the Bradford method):

1 - free enzyme (urease); 2 - enzyme immobilized on the lateral surface of the spectrophotometric cell and contained in the microcells of the sensitive coating with the architecture of the PAAG- (PSS-PAGE) 2 shell.

Study medium: 1 mM MES, 100 mM NaCl, initial pH 6.0

Thus, it has been shown that by potentiometric method using a new type of polymeric urease sensor on a glass pH electrode it is possible to measure urea concentrations ranging from 10-20  $\mu\text{M}$ . The upper limit of the measurement depends on the concentration of urease placed in the polyelectrolyte coating and on the properties of the electrode. We were able to measure more than 100 mM urea. In fact, when developing a urea sensor for medical diagnosis, it is not necessary to measure such high urea concentrations, since the normal urea content in the human blood is between 1.8 and 7.5 mM, depending on the age.

Studies of the stability of a new type of urea sensor showed that when stored in distilled water at a temperature of 4  $^{\circ}\text{C}$ , the sensor is capable of operating for up to 2 months. At the same time, the decrease in activity of immobilized urease is initially 40-50%. The stability of the sensor over time can, among other things, be due to the stability of the polyelectrolyte shells that protect the enzymes from the external environment in the microcells of the coating. Perhaps in this case, the size of the microcapsules, as well as the number of polyelectrolyte layers forming the microcapsule shell, will be important for increasing the stability of the polyelectrolyte coating. We carried out preliminary studies of the strength of 10  $\mu\text{m}$  microcapsules containing a calcium-carbonate core with the help of a NanoScan-4D nanodimeter [20]. It was shown that the destruction of a single microcapsule occurred when it was compressed by 1.1  $\mu\text{m}$  and a load value of 25 mN. Investigation of the strength of microcapsules with a remote calcium carbonate nucleus depending on the size of microcapsules and the number of layers of capsule shell polyelectrolytes is of interest for improving the stability of microcapsules and a new polyelectrolyte coating.

The necessary component of research in the development of biosensors is testing on biological fluids. Investigations of the urease polymer sensor for the determination of urea in biological fluids were conducted using urine and blood as an example. These experiments are presented in [9], from which it follows that if the accuracy of measuring by our method the concentration of urea in daily urine diluted 100 times approaches the error obtained during dilution of urine, then a different picture is observed when measuring urea in serum. Blood in different people has its own pH and buffer capacity, so when measuring urea in blood serum, we tested the "double additives" method, which increased the accuracy of measurements to 5%.

The sensitivity of the urease sensor can be significantly increased by using pH-sensitive field effect transistors (figure 5).

As can be seen from the figure, the field effect transistor has increased the sensitivity of the sensor by more than an order of magnitude. The enzyme consumption during the creation of a sensitive field-transistor coating was 20  $\mu\text{l}$  from the 3 ml set (see Materials and Methods). This amount of enzyme is used for one or two measurements in enzyme analysis by the usual spectral method in polyclinics.

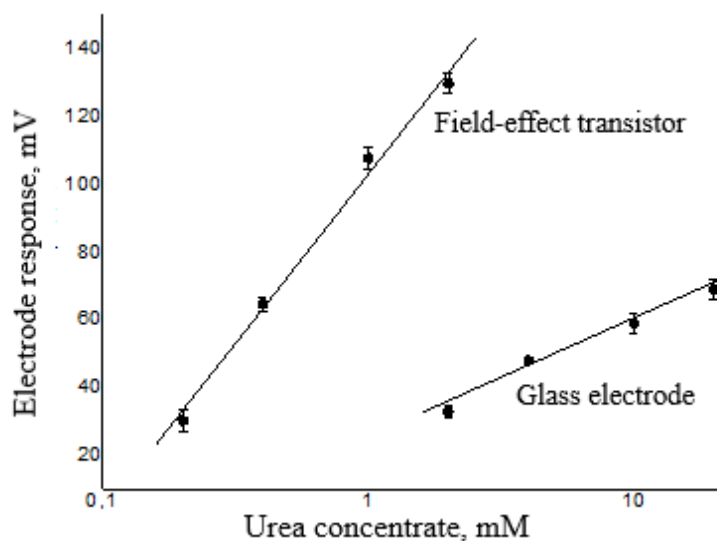


Figure 5 – Comparison of the response of the pH-sensitive field-effect transistor and the glass pH electrode to the urea concentration.

The sensitive coating is applied to the glass electrode ball and to the surface of the recording element of the transistor. Study medium: 2 mM Tris-HCl, 200 mM NaCl, initial pH 7.8

**Conclusion.** The urea biosensor manufactured with the help of polymer technologies and representing a combination of polyelectrolyte layers and microcapsules with an enzyme inside and a shell of the same polyelectrolytes, as shown by the experimental data, is perfectly suitable for determining the urea concentration in blood and urine. The technology of manufacturing an enzymatic biosensor does not differ significantly from the known technology of manufacturing microcapsules with an enzyme by the layer-by-layer method [4-6]. This allows us, when constructing a biosensor, to use the information obtained on encapsulated enzymes by other authors. In this case, the urea biosensor is able to work for a long time (up to 2 months) without significant loss of enzyme activity. One of the significant results of this work from our point of view is two factors. The first factor is the optimization of the conditions for the production of a functionally active enzyme immobilized in a polyelectrolyte coating, when the enzyme after the immobilization procedure shows an activity comparable to that of a freshly prepared free enzyme. Such a result will allow reducing the cost of enzymes when creating a sensitive layer of the developed urea analyzer. And the second factor is that the polymer coating with the enzyme is able to work not only as an enzyme electrode, but also as an enzyme microreactor, without decreasing the rate of signal registration after passing the catalytic urease-urea reaction. This is due to the fact that the layers of polyelectrolytes separating the enzyme from the external analyte solution have a nanometer thickness and are easily permeable to urea and decomposition products of the urease-urea catalytic reaction. Separation of the sensitive sensor from the recording electrode provides many opportunities for designers of urea analyzers based on a polymer ultrathin coating.

**Acknowledgments.** Work was accomplished by grant financing of the Committee of Science of the Ministry of Education and Science of the Republic of Kazakhstan by AP05134201 project.

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### **ҚАН ЖӘНЕ НЕСЕПТІ ТАЛДАУ ҮШІН ПОЛИМЕРЛІ ТЕХНОЛОГИЯЛАРДЫ ПАЙДАЛАНУ АРҚЫЛЫ МОЧЕВИНА БИОДАТЧИГІН ЖАСАУ**

**Аннотация.** Полимерлі нанотехнологиялар негізінде сұйықтықтарда мочевианы анықтай алатын ферментті тіркеуіштер мен микрореакторлар жасалды. Ферментті тіркеуішті жасау технологиясы laeg-by-laeg әдісімен ферментті микрокапсулалар жасаудың белгілі технологиясынан айтарлықтай ерекшеленбейді. Бұл бізге басқа авторлармен инкапсуляцияланған ферменттерден алынған мәліметтерін биотіркеуіш жасауда ақпарат ретінде мүмкіндік береді. Мочевина биосенсоры ұзақ уақыт бойы ферменттің белсенділігін айтарлықтай жоғалтпай ұзақ уақыт бойы (2 айға дейін) жұмыс жасай алатындығы табылды. Полимерлі технология басқа да ұқсас әдістерге карағанда жеңіл және арзан болып табылады. Биологиялық сұйықтарды (қан, несеп) экспресс анықтау үшін полимерлі ферментті чипі бар мочевиана анализаторы ұсынылады. Бұл жұмыста біздің ойымызшайтарлықтай екі артықшылық факторы бар. Бірінші фактор – полиэлектрлитті жабынға иммобилизацияланған функционалды-белсенді фермент алу жағдайын оңтайландыру, мұнда иммобилизация әрекетінен кейін фермент жаңа даярланған бос ферменттің белсенділігіне ұқсас белсенділік көрсетеді. Мұн-

дай нәтиже мочевины анализаторы қондырғысын жасауда сезімтал қабатты дайындауда ферменттерге кететін шығындарды арзандатады. Екінші фактор, ферменті бар полимерлі жабын ферментті электрод ретінде ғана жұмыс жасап қоймай, уреазы-мочевина каталитикалық реакциясы өткеннен кейін тіркеу жылдамдығын төмендетпей ферментті микрореактор ретінде де іс атқарады.

**Түйін сөздер:** ферментті биосенсорлар, полимерлі наноматериал, портативті анализатор, микрореактор, микрокапсулалар, мочевины.

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### **РАЗРАБОТКА БИОДАТЧИКА МОЧЕВИНЫ С ПРИМЕНЕНИЕМ ПОЛИМЕРНЫХ ТЕХНОЛОГИЙ ДЛЯ АНАЛИЗОВ КРОВИ И МОЧИ**

**Аннотация.** На основе полимерных нанотехнологий созданы ферментные датчики и микрореакторы, способные определять мочевины в жидкостях. Технология изготовления ферментного биодатчика существенно не отличается от известной технологии изготовления микрокапсул с ферментом методом *layer-by-layer*. Это позволяет нам при конструировании биодатчика пользоваться информацией, полученной на инкапсулированных ферментах другими авторами. Показано, что биосенсор мочевины способен работать в течение длительного времени (до 2 месяцев) без значительной потери активности фермента. Полимерная технология изготовления датчиков менее трудоемкая и дорогостоящая по сравнению с другими аналогичными технологиями. Предлагаются к разработке биосенсорные приборы – анализаторы мочевины с полимерными ферментными чипами для экспресс-диагностики биологических жидкостей (кровь, моча). Одним из существенных результатов настоящей работы с нашей точки зрения являются два фактора. Первый фактор – это оптимизация условий получения функционально-активного фермента, иммобилизованного в полиэлектролитное покрытие, когда фермент после процедуры иммобилизации показывает активность сравнимую с активностью свежеприготовленного свободного фермента. Такой результат позволит удешевить расходы на ферменты при создании чувствительного слоя разрабатываемого прибора-анализатора мочевины. И второй фактор, это то, что полимерное покрытие с ферментом способно работать не только как ферментный электрод, но и как ферментный микрореактор, при этом не уменьшая скорость регистрации сигнала после прохождения каталитической реакции уреазы-мочевина.

**Ключевые слова:** ферментные биосенсоры, полимерный наноматериал, портативный анализатор, микрореактор, микрокапсулы, мочевины.

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**ISSN 2518-1629 (Online), ISSN 2224-5308 (Print)**

<http://www.biological-medical.kz/index.php/ru/>

Редактор *М. С. Ахметова, Т. М. Апендиев, Д. С. Аленов*  
Верстка на компьютере *Д. Н. Калкабековой*

Подписано в печать 06.12.2018.

Формат 60x881/8. Бумага офсетная. Печать – ризограф.  
4,5 п.л. Тираж 300. Заказ 5.