ISSN 0233–7657. Biopolymers and Cell. 2014. Vol. 30. N 4. P. 291–298 doi: http://dx.doi.org/10.7124/bc.0008A3

UDC 577.15.08:543.9

Elaboration of new method of enzyme adsorption on silicalite and nano beta zeolite for amperometric biosensor creation

O. O. Soldatkin[1, 2], B. Ozansoy Kasap[3], B. Akata Kurc[3, 4], A. P. Soldatkin[1, 2], S. V. Dzyadevych[1, 2], A. V. El'skaya[1]

[1]Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine
150, Akademika Zabolotnoho Str., Kyiv, Ukraine, 03680

[2]Institute of High Technologies, Taras Shevchenko National University of Kyiv
64, Volodymyrska Str., Kyiv, Ukraine, 01601

[3]Central Laboratory, Middle East Technical University
Ankara, Turkey, 06531

[4]Micro and Nanotechnology Department, Middle East Technical University
Ankara, Turkey, 06531

alex_sold@yahoo.com

Aim. *Optimization of a new method of enzyme immobilization for amperometric biosensor creation.* ***Methods***. *The amperometric biosensor with glucose oxidase immobilized on zeolites as bioselective elements and platinum disk electrode as transducers of biochemical signal into the electric one was used in the work.* ***Results***. *The biosensors based on glucose oxidase adsorbed on zeolites were characterized by a higher sensitivity to glucose and a better inter-reproducibility. The best analytical characteristics were obtained for the biosensors based on nano beta zeolite. It has been found that an increase in the amount of zeolite on the surface of amperometric transducer may change such biosensor parameters as sensitivity to the substrate and duration of the analysis.* ***Conclusions***. *The proposed method of enzyme immobilization by adsorption on zeolites is shown to be quite promising in the development of amperometric biosensors and therefore should be further investigated.*

Keywords: Biosensor, amperometric transducer, enzyme adsorption, silicalite, nano beta zeolite, glucose oxidase.

Introduction. It is well known that enzyme immobilization plays a key role in the development of biosensors. In recent years, the study and optimization of the methods of immobilization have attracted considerable interest of researchers. Special additives injected in the sensitive membrane upon immobilization can improve the sensitivity and stability of the immobilized enzyme [1]. Recently a great deal of attention has been paid to the immobilization of proteins on nanoparticles, in particular zeolites, which are able to retain the biological activity of proteins [2].

Zeolites are an important group of minerals for industrial and other purposes. They combine rarity, complexity and unique crystal habits [3]. Formed in cracks or cavities of volcanic rocks, zeolites are a result of a very slow progressive metamorphism. Some of them, which are formed due to barely perceptible heat and pressure, can be called metamorphic only conditionally whereas others are found in obviously metamorphic regimes. Zeolites are of great interest due to their large surface areas, rigid and well defined pore structures, thermal stability, and tailorable surface charges with respect to other types of nanomaterials [4]. Particularly, the nanosized pores of zeolites can be adjusted to precisely determined uniform openings allowing the molecules smaller than the pores diameter to be adsorbed. Various size of pores in synthetic zeolites opens up a wide range of possibilities in terms of sieving molecules of different

size or shape from gases and liquids [2, 5]. Finally, zeolites are known to be stable under both wet and dry conditions and well-tolerated by microorganisms, which provides an enhanced compatibility with biochemical analyses [2]. All these properties make zeolites unique nanomaterials and promising candidates for the immobilization of biological molecules and for the advanced analytical tasks.

At present, some variants of biosensors containing zeolite crystals are known. Zeolites can be embedded into bioselective elements to improve analytical characteristics of biosensors, *i. e*. their sensitivity to the substrate, linear and dynamic ranges, signal inter- and intra-reproducibility. In [6–8] the use of zeolite in the biosensor structure has been shown to increase the sensitivity and improve the selectivity. As demonstrated in [4], zeolites of various kinds can be effectively applied for the glucose oxidase immobilization while developing glucose amperometric biosensors to optimize their sensitivity, selectivity and stability. It was also shown that zeolites can be a basis for the creation of a new type of amperometric biosensors without mediators since zeolites can serve as charge carriers [7, 9]. As reported in [10, 11], zeolites are used in conductometric biosensors as alternative carriers for the enzyme immobilization. Diverse variants of co-immobilisation of urease and BEA-zeolites onto the surface of conductometric transducers were analyzed in respect to the improvement of analytical characteristics of biosensors for urea determination [11]. A similar increase in sensitivity of bioselective elements was obtained when using BEA-zeolites in biosensors based on pH-sensitive field effect transistors [12–14]. These studies have shown that the greatest effect of improving the sensitivity of potentiometric biosensors is observed with the biomembranes of complex architecture [13]. The promising results were obtained when clinoptilolite was used at the development of enzyme biosensors based on pH-sensitive field-effect transistors [15–18]. The basic idea was to attain high sensitivity of the potentiometric transducer to NH_4^+ by deposition of a clinoptilolite layer on the transducer surface [15]. The described effect was also observed in the biosensors based on urease [17] and urease co-immobilized with arginase [18]. Another option of the use of nanoscale materials in the design of biosensors has been proposed in [19], namely silicalite as a carrier for the enzyme sorption. The biosensors obtained were characterized by a significantly higher signal reproducibility.

The goal of this study was to check a possibility of using silicalite and nano beta zeolite for creation of amperometric glucose biosensor with enhancement analytical characteristics.

Materials and methods. *Materials*. Glucose oxidase (GOD, EC 1.1.3.4) from *Aspergillus niger* with activity 272 U/mg («Genzyme», UK) was used in biorecognition elements of biosensors. Bovine serum albumin (BSA, fraction V), glucose, glycerol, ascorbic acid, HEPES, and 50 % aqueous solution of glutaraldehyde (GA) have been received from «Sigma-Aldrich Chemie» (Germany). All other chemicals were of p. a. grade.

Synthesis of zeolite crystals. S i l i c a l i t e. To synthesize the silicalite solution we used 1TPA-OH : 4 TEOS : $350 \times H_2O$. When hydrolysing tetraethoxysilane (TEOS) with tetrapropylammonium hydroxide (TPA-OH) we obtained a homogeneous solution by constant stirring for 6 h at room temperature. The crystallization took place at 125 °C during one day. The material, which did not react, was removed from the solution by centrifugation. The size of silicalite particles was approximately 400 nm.

N a n o b e t a z e o l i t e. The molar composition of the nano beta zeolite is 0.25 Al_2O_3 : 25 SiO_2 : 490 H_2O : : 9 TEAOH. Silica source was TEOS (98 %, «Aldrich»). Aluminum isopropoxide (98 %, «Aldrich»), tetraethylammonium hydroxide (TEA-OH) (20 wt.% in water, «Aldrich») and doubly distilled water were used as the other reactants. Aging was continued under static conditions for 4 h with clear solution. The crystallization was completed within 17 days under static conditions at 100 °C in teflon lined autoclaves. The product was purified using centrifugation [20]. Approximate size of the nano beta zeolite particles is 60 nm.

Characterization of zeolites. The resulting samples were characterized by powder X-ray diffraction (XRD) using Ni filtered Cu-Kα radiation in a Philips PW 1729. Scanning electron microscopy (SEM) analysis were performed after AuPb coating in a 400 Quanta FEI. The energy dispersive X-ray spectroscopy (EDX) analyses of the all samples were carried out utilizing a Phoenix EDAX X-ray analyzer equipped with Sapphire super ultrathin window detector attached to the Hitachi S-4700 FE-SEM. The nitrogen adsorption/desorp-

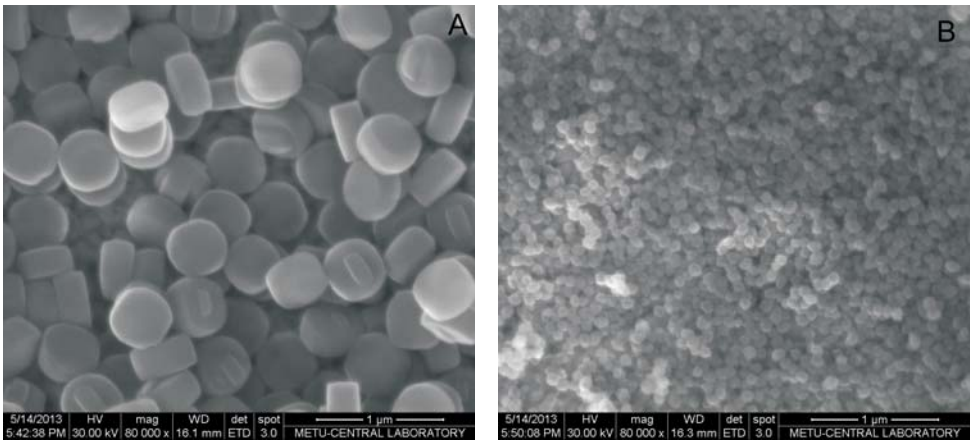

Fig. 1. Scanning electron microscope image of silicalite (*A*) and nano beta zeolite (*B*)

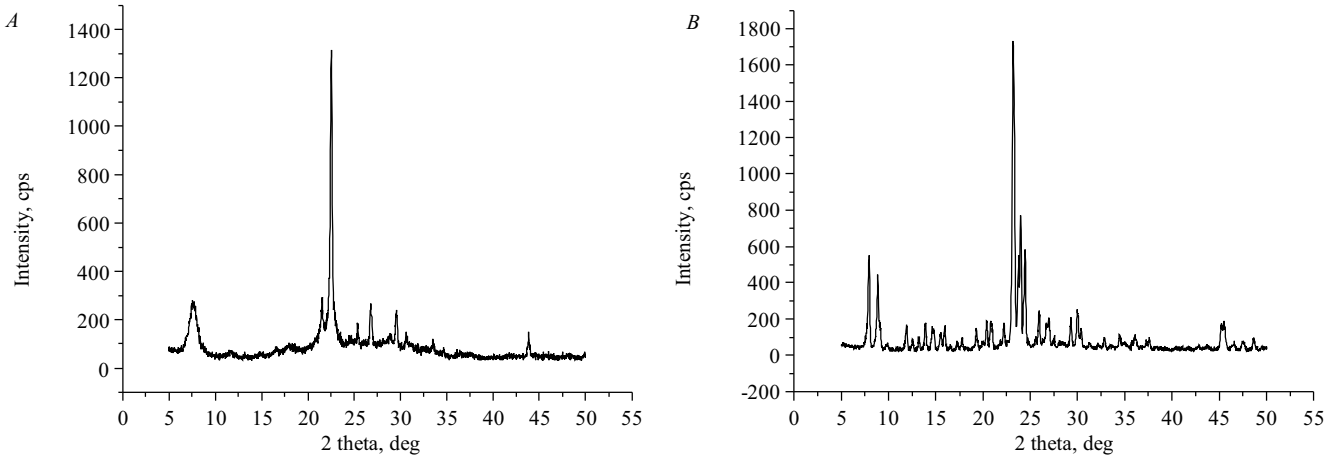

Fig. 2. XRD spectrum of synthesized zeolites: *A* – nano beta zeolite; *B* – silicalite

tion experiments were carried out at by NOVA 3000 series («Quantachrome Instruments», USA) instrument. Surface area of the samples were obtained by Multipoint BET, while the pore size and pore volumes were obtained by Saito-Foley (SF) and t-plot methods. A sample preparation method includes outgassing samples under vacuum at 300 K for 4 h before analysis. The morphologies of the produced silicalite and nano beta zeolite can be seen in Fig. 1.

According to the X-ray diffraction data presented in Fig. 2, all samples exhibited the characteristic diffraction lines of their structures. In Table, Si/Al ratios, particle sizes, pore sizes, external and total surface area, micro- and mesopore volume are given.

Design of amperometric transducers. Most of existing amperometric systems use wire electrodes, which are structurally rather inconvenient for biosensors [21, 22]. It is much more promising to utilize planar thin-film electrode systems placed on a single glass or a ceramic substrate. This is especially true for the multisensor systems. For the development of a planar amperometric multisensor, the design was chosen, in which the electrochemical cell consisted of four working electrodes, reference and auxiliary electrodes.

Enzyme immobilization in glutaraldehyde vapour. GA is a polyfunctional agent which forms covalent bonds between biocatalytic particles or proteins. Therefore, the enzyme immobilization with glutaraldehyde is often used for the development of enzyme biosensors [23]. This immobilization method produces a three-dimensional matrix, in which the enzyme is closely trapped with the electrode material, thus improving both retention of the biomolecule on the electrode surface and electrical communication.

To produce a working bioselective membrane, a mixture of 5 % glucose oxidase (GOX), 5 % BSA, 10 %

Characteristics of zeolites

Sample name	Si/Al[a]	Part. size (nm)[b]	Pore size (nm)[c]	Sext (m^2/g)[d]	Stotal (m^2/g)[e]	Pore volume (cc/g)c
Nano beta zeolite	26.54	~ 60	0.43	190	472	0.20
Silicalite	No Al	~ 400	0.45	52	185	0.08

N o t e. Measured by: [a]EDX; [b]SEM; [c]Saito-Foley (SF) method; [d]t-plot method; [e]BET.

glycerol was prepared in 20 mM phosphate buffer, pH 7.2. This mixture was deposited on the transducer surface using Eppendorf microsampler (total volume 0.1–2.5 μl) until the working surface is covered completely. The volume of each membrane was about 0.05 μl. All membrane mixtures contained the same total amount of protein.

Afterwards, the transducers with membrane mixtures were placed in saturated vapor of GA for 15–25 min, dried in the air at room temperature for 15 min, and prior to usage washed with the buffer solution from unbound GA.

Dip-coating zeolite onto transducers. A zeolite layer was formed on the transducer surface by dip-coating. 10 % zeolite solution in 5 mM PBS, pH 6.5, was used. 0.4 μl of the solution was deposited onto all active zones of multitransducer, then it was heated during 3 min to 150 °C. This temperature had no effect on silicalite and did not influence the transducer working parameters.

Modification procedure for zeolite monolayers. Firstly, we tried to attach zeolite to the electrodes surface but failed in our attempts. Then, we used a layer of poly(ethyleneimine) (PEI) between zeolite and electrode surface. In this case, the zeolite attachment was attained but there appeared a problem of homogeneity. To solve it, we used mucasol (1/6 v/v) in distilled water, which gave good results due to changing the surface hydrophility.

The electrode surfaces were dip-coated with mucasol for 15 min, rinsed with copious amount of distilled water and dried under air. For the formation of homogeneous layers of PEI, both dip-coating and spin-coating techniques had been tried. Since spin-coating gave more homogeneous layers, it was used further on. The effects of PEI solvent type (hot water and ethanol), PEI concentration (0.5, 1, 3, 5 %), spin-coating time (3000 rpm 15 s, 7 s), calcination temperature (100, 90, 50 °C) were investigated. The obtained monolayers were checked with microscope. The suitable conditions for zeolite monolayer production were chosen as follows: spin-coating with 0.5 % PEI in ethanol at 3000 rpm during 15 s, and calcination at temperature 90 °C for 30 min.

The synthesized zeolites were directly attached to the obtained electrode surfaces simply by rubbing zeolites with a finger, a technique called direct attachment. These electrodes were used in further studies.

Enzyme adsorption on zeolites. 0.1 μl of 5 % GOD solution in 20 mM phosphate buffer, pH 6.5 was deposited onto the active zones of multitransducer previously coated with silicalite, then the transducer was exposed to complete air-drying (for 20 min). Neither GA nor any other auxiliary compounds were used. Next, the transducers were submerged into the working buffer for 20–30 min to wash off the unbound enzyme. After experiments, the transducer surface was cleaned from enzyme with ethanol-wetted cotton.

Experimental setup for amperometric measurements. A three-electrode scheme of amperometric analysis was used. The working amperometric transducers were developed, which were connected to the Palm Sens potentiostat (Netherlands) along with the auxiliary nickel electrode (with a much larger area of the nickel surface compared to the working electrode) and the Ag/AgCl reference electrode.

Each electrode has its own function in the amperometric analysis. When positive potential is applied to the working electrode, all the molecules in solution on the electrode surface are oxidized and an electron transition from the solution to the electrode takes place. If there was no additional electrode, a large potential difference would be generated due to the stoichiometric imbalance. The function of the auxiliary electrode is to form the external circuit providing the electrons a pathway back to the solution. Obviously, this results in the reduction process on the auxiliary electrode, equivalent to the oxidation process on the working electrode. This flow of electrons generates a current in the amperometric sensor. The third electrode is a reference electrode, which should contain a known chemical compound, which in-

cludes both forms of the redox pair. Usually it is either $Hg/HgCl_2$ (saturated calomel electrode) or Ag/AgCl (chloro-silver electrode). Since the applied potential is fixed, the reference electrode has a stable point, which can be also fixed on the working electrode for measurement. That is, the applied potential is controlled between the working and reference electrodes, whereas the current is measured between the working and auxiliary electrodes [24].

All measurements were carried out in an open measuring cell with permanent stirring at a constant potential of +1 V vs Ag/AgCl reference electrode.

Measurement procedure. Measurements were carried out in 20 mM HEPES, pH 7.4, in a voltamperometric mode at a constant potential of +1 V vs Ag/AgCl reference electrode in an open cell with vigorous stirring. The substrate concentration in the measuring cell was specified by the introduction of aliquots of the substrate standard stock solution to the working buffer. All experiments were performed in at least three series.

Results and discussion. The main goal of this research was to develop a new amperometric biosensor with enhanced analytical characteristics by using a novel method of immobilization of the enzyme based on its adsorption on zeolites. This method was first proposed for conductometric biosensors [19]. In this work we presented our attempt to use the mentioned method and to upgrade it for the amperometric transducers. The enzyme GOD was chosen as one of the most stable and studied enzymes.

The operation of amperometric biosensor for glucose determination is based on the enzymatic reaction with consequent hydrogen peroxide oxidation on the working electrode, which occurs when applying necessary potential and can be directly registered by the amperometric transducer. In the presense of glucose, the reactions take place on the electrode surface as follows:

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2; \quad (1)$$

$$H_2O_2 \xrightarrow{+\,1000\ \text{mV}} 2H^+ + 2e^-. \quad (2)$$

In this case, the biosensor response is proportional to the glucose concentration. At first, we fabricated a number of biosensors based on GOD immobilized by the method of enzyme adsorption on silicalite proposed in [19]. In details the immobilization technique is described in «Materials and Methods». The biosensors sensitivity to glucose was tested. The results obtained did not justify our expectations – it appeared that, along with good sensitivity, the biosensor responses were very slow and so biosensors lost one of their main advantages – fast analysis. For example, the response to 1 mM glucose amounted 40 min. We believe it is an effect of too thick layer of silicalite formed on the transducer surface by dip-coating. However, we considered that the value of response of the biosensors based on GOD adsorbed on silicalite was 3 times more than that of the biosensors based on the traditional methods of GOD immobilization in GA vapor. Therefore, we decided to modify the method of zeolite application on the surface of amperometric transducer in order to obtain a monolayer of zeolite. In details the technique proposed is described in «Materials and Methods». Using this technique we created two types of biosensors based on silicalite and nano beta zeolite. The characteristics of these biosensors were compared with those of the biosensors based on the traditional immobilization in GA vapor.

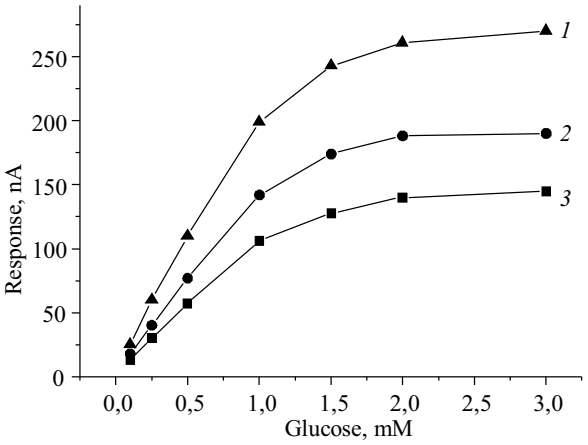

Fig. 3. Calibration curves for glucose detection by biosensors based on GOD adsorbed on nano beta (*1*) and silicalite (*2*), and GOD immobilized in GA vapor (*3*). Measurements were carried out in 5 mM HEPES buffer, pH 7.2

Since the sensitivity and linear range of measurement are the most important working characteristics of any biosensor, we investigated an influence of different types of immobilization on these parameters. The calibration curves were obtained for each biosensor. The linear ranges of measurement for all biosensors were identical, whereas sensitivities differed. As shown in Fig. 3,

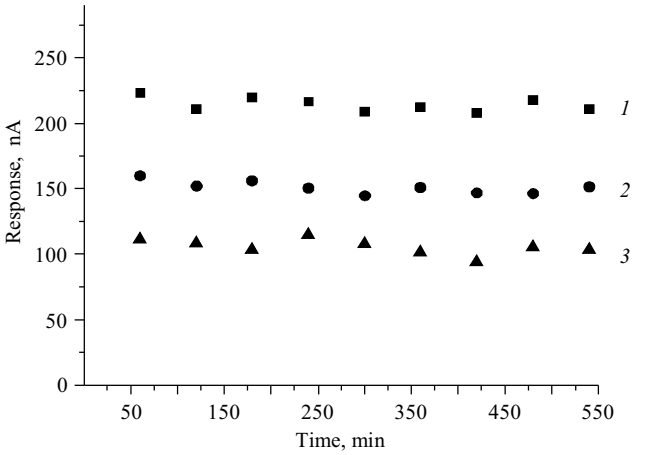

Fig. 4. Reproducibility of responses of biosensors based on GOD adsorbed on nano beta (*1*) and silicalite (*2*), and GOD immobilized in GA vapor (*3*). Glucose concentration – 1 mM. Measurements were carried out in 20 mM HEPES buffer, pH 7.2

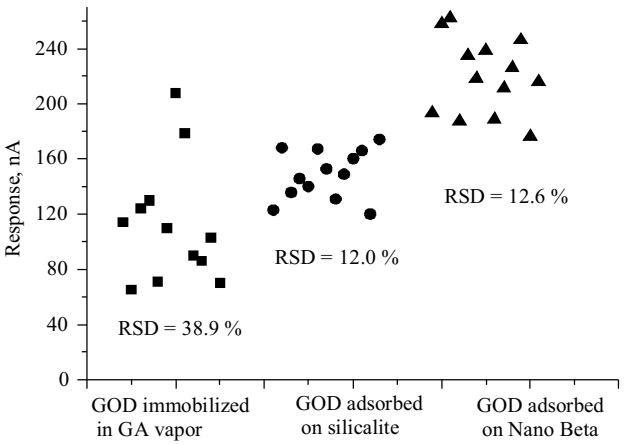

Fig. 5. Reproducibility of preparation of biosensors based on GOD adsorbed on nano beta (*1*) and silicalite (*2*), and GOD immobilized in GA vapor (*3*). Glucose concentration – 1 mM. Measurements were carried out in 20 mM HEPES buffer, pH 7.2

the biosensors based on using both zeolites were characterized by a higher sensitivity than the biosensors based on the GOD immobilized in GA vapor.

It is known, that one of the most important biosensor characteristics is the reproducibility of the biosensor signal during operation. Therefore, it was necessary to explore this option for the biosensors with the GOD adsorbed on monolayers of zeolites and compare it with that for the biosensors based on the GOD immobilized in GA vapor.

At the next stage, we studied reproducibility of responses of all three biosensors during several hours of continuous operation. One measurement of glucose took 3–5 min. The interval between measurements was about 20 min, during this time we washed the biosensors from substrates by working buffer. We did not reveal any considerable decrease in responses of all three sensors after 10 subsequent measurements. As seen (Fig. 4), all biosensors demonstrated high signal reproducibility. The relative standard deviation of responses to glucose was similar for these biosensors (about 8 %).

Since the reproducibility of biosensor manufacturing is a well-known challenge in biosensorics, we should prove that this characteristic for the proposed by us procedure of enzyme immobilization, *i. e*. the enzyme adsorption on zeolite monolayer, is not worse than for the immobilization in GA vapor. Therefore, we checked reproducibility of manufacturing of all three biosensors (with and without zeolite) and compared the results obtained (Fig. 5). As can be seen, the biosensors based on the GOD adsorption on nano beta zeolite had better reproducibility as compared to the biosensors with the GOD adsorbed on silicalite, whereas the variant of enzyme immobilization in GA vapor was the worst.

Summing up all the experiments conducted, we revealed that the biosensors based on the GOD adsorbed on nano beta zeolite had the best parameters. This type of immobilization was the most stable and reproducible, and the biosensors were characterized with the highest and fastest responses. However, though the promising results were obtained, we made further endeavour for improving the biosensors sensitivity by an increase in the number of nano beta zeolite particles on the electrode surface. It is known that, increasing the polymer concentration in course of applying zeolite monolayers, it is possible to increase an amount of zeolite on the surface of amperometric transducer. Nevertheless, as it was shown at the beginning of the work, the zeolite layer formed on the transducer surface by dip-coating procedure is too thick, which results, along with good sensitivity, in essential increase in the analysis time. Therefore, a compromise was required, *i. e*. it was necessary to find the immobilization conditions, which would ensure the greatest sensitivity to the substrate and short analysis time. The results of this study are shown in Fig. 6. As can be seen at a higher polymer concentration used in the procedure of zeolite application, the value of biosensor response increases, but the response time increa-

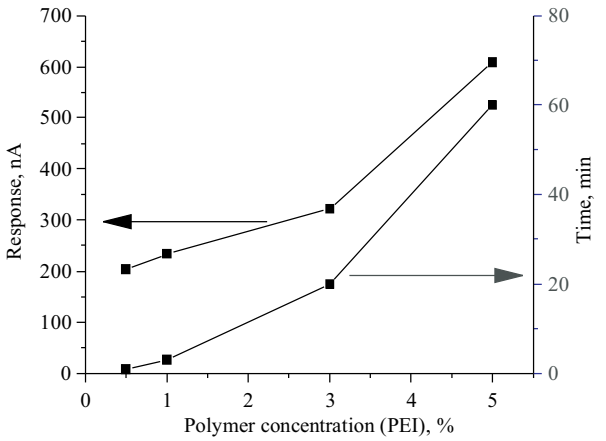

Fig. 6. Dependences of value (*1*) and time (*2*) of biosensor response on different concentration of polymer on amperometric electrode surface. Glucose concentration – 1 mM. Measurements were carried out in 20 mM HEPES buffer, pH 7.2

ses as well. That is why we choose 0.5 % PEI for zeolite monolayer creation. In this case, we obtained enough high response and short response time.

Conclusions. The enzyme adsorption on zeolites has been verified as a method of immobilization of biological membranes for the development of amperometric biosensors with improved analytical characteristics. For comparison the most common traditional method of immobilization in glutaraldehyde vapor has been chosen. The best results were obtained for the biosensor based on immobilization with nano beta zeolite particles, the worst – for the biosensors based on the immobilization in glutaraldehyde. Besides sensitivity, stability and other analytical characteristics, it has been shown that the proposed biosensor based on nano beta zeolite particles is characterized by good repeatability of responses (error did not exceed 8 %) and reproducibility of biosensor manufacturing (error did not exceed 13 %).

It has been shown that the proposed method of the enzyme immobilization by adsorption on zeolites is quite promising for further application in the development of amperometric biosensors.

Funding. The authors gratefully acknowledge the financial support of this study by Project European IRSES-318524-NANODEV. Furthermore, this study was partly supported by NASU in the frame of Scientific and Technical Program «Sensor systems for medico-ecological and industrial-technological requirement: metrological support and experimental operation».

Розробка нового методу на основі адсорбції ферменту на силікаліті та нано-бета цеоліті для створення амперометричних біосенсорів

О. О. Солдаткін, Б. Озансой Касап, Б. Аката Курк, О. П. Солдаткін, С. В. Дзядевич, Г. В. Єльська

Резюме

Мета. *Оптимізація нового методу іммобілізації ферментів для розробки амперометричних біосенсорів.* ***Методи***. *Використано іммобілізовану глюкозооксидазу на цеоліті як біоселективний елемент біосенсора та платиновий дисковий електрод – як амперометричний перетворювач біохімічного сигналу в електричний.* ***Результати***. *Біосенсор на основі глюкозооксидази, адсорбованої на цеолітах, вирізняється високою чутливістю до глюкози та покращеною інтер-відновлюваністю виготовлення біосенсорів. Найкращі аналітичні характеристики притаманні біосенсору на основі нано-бета цеоліту. Встановлено, що при зміні кількості цеолітів на поверхні амперметричного перетворювача можна варіювати параметри біосенсора, такі як чутливість до субстрату та час аналізу.* ***Висновки***. *Показано, що запропонований метод іммобілізації, а саме – адсорбція ферментів на цеолітах є дуже перспективним при розробці амперометричних біосенсорів.*

Ключові слова: біосенсор, амперометричний перетворювач, адсорбція ферментів, силікаліт, нано-бета цеоліт, глюкозооксидаза.

Разработка нового метода на основе адсорбции фермента на силикалите и нано-бета цеолите для создания амперометрических биосенсоров.

А. А. Солдаткин, Б. Озансой Касап, Б. Аката Курк, А. П. Солдаткин, С. В. Дзядевич, А. В. Ельская

Резюме

Цель. *Оптимизация нового метода иммобилизации ферментов для разработки амперометрических биосенсоров.* ***Методы***. *Использовали иммобилизованную глюкозооксидазу на цеолитах как биоселективный элемент биосенсора и платиновый дисковый электрод – как амперометрический преобразователь биохимического сигнала в электрический.* ***Результаты***. *Биосенсор на основе глюкозооксидазы, адсорбированной на цеолитах, отличается высокой чувствительностью к глюкозе и улучшенной интер-воспроизводимостью приготовления биосенсоров. Наилучшими аналитическими характеристиками обладает биосенсор на основе нано-бета цеолита. Установлено, что при изменении количества цеолитов на поверхности амперометрического преобразователя можно менять параметры биосенсора, такие как чувствительность к субстрату и время анализа.* ***Выводы***. *Показано, что предложенный метод иммобилизации, а именно – адсорбция ферментов на цеолитах является перспективным при разработке амперометрических биосенсоров.*

Ключевые слова: биосенсор, амперометрический преобразователь, адсорбция ферментов, силикалит, нано-бета цеолит, глюкозооксидаза.

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Received 15.05.14