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Application of silicalite for improvement of enzyme adsorption on the stainless steel electrodes

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Aim. *Improvement of analytical characteristics of an enzyme biosensor based on new inexpensive perspective stainless steel electrodes using silicalite nanoparticles.* ***Methods***. *Conductometric enzyme biosensor was used.* ***Results***. *Three methods of glucose oxidase (GOx) immobilization were studied and compared: GOx adsorption on silicalite modified electrodes (GOx-SME); cross-linking by glutaraldehyde without silicalite (GOx-GA); GOx adsorption on SME along with cross-linking by glutaraldehyde (GOx-SME-GA). The GOx-SME-GA biosensors based on stainless steel electrodes were characterized by 12–25-fold higher sensitivity comparing with other biosensors. The developed GOx-SME-GA biosensors were characterized by good reproducibility of glucose biosensors construction (relative standard deviation (RSD) – 18 %), improved signal reproducibility (RSD of glucose determination was 7 %) and good storage stability (29 % loss of activity after 18 days).* ***Conclusions***. *The method of enzyme immobilization using silicalite together with GA cross-linking sufficiently enhances the enzyme adsorption on the stainless steel electrodes and improves the analytical parameters of biosensors. This method is found to be promising for further creation of other enzyme biosensors.*

Keywords: enzyme immobilization, silicalite, glucose oxidase, conductometric transducer, biosensor.

Introduction. Nowadays the enzyme adsorption on solid surfaces is widely used in many fields, which are generally referred to biotechnology, environmental science/engineering, biomedicine, microbial synthesis; in particular, it plays a key role in biosensors production. Physical adsorption on a certain carrier is the oldest and the simplest method of enzyme immobilization. Enzyme adsorption usually implies neither additional chemical reagents nor activation; therefore, this is the least denaturing method of immobilization, which provides good retention of the enzyme activity. Besides, the adsorption is commercially attractive due to a lower cost of its performing as compared with other immobilization methods. In the past few decades, the biomolecules immobilization using different nanomaterials became one of the most common approaches in the immobilization techniques [1]. Zeolites were found to be suitable for this aim, due to their properties [2–4].

Zeolites are hydrated microporous crystalline aluminosilicates. They are composed mainly of silicon, aluminum and oxygen. The modification of crystal structures makes it possible to obtain zeolites with different

properties. The zeolites micropores create a vast and regular network of channels and cages with well-defined sizes and shapes. Furthermore, zeolites are able to exchange ions with some compounds. They are also selective adsorbers [5–7].

The conductometric biosensors demonstrate several advantages over other electrochemical biosensors, namely: electrodes miniaturization and large scale production by inexpensive technology are possible; noble metals can be substituted for cheaper ones, *e. g*. Ni; a reference electrode is not needed; a light sensitivity is absent; small driving voltage decreases a power consumption; a wide range of substances can be determined using appropriate reactions and mechanisms [8].

A number of zeolite-based biosensors have been described previously. Silicalite recently has been used for the creation of amperometric biosensor based on glucose oxidase [9]. It has been found that the sensitivity and response time of the developed biosensors depend on the amount of silicalite on the transducer surface. The usage of natural zeolite clinoptilolite in the bioselective membrane of the conductometric biosensor for urea determination has been described [10]. The optimal zeolite concentration in nanobiocomposites, which permits to extend the linear measurement range without any loss in the sensitivity to urea, has been found to be 1.5 % [11]. The changing of immobilization procedure using zeolites for the urea biosensor construction include an addition of different types of zeolites to the immobilization mixture for modification of the standard cross-linking procedure with glutaraldehyde as well as the urease adsorption without glutaraldehyde [12]. In another work, the urea and butyrylcholine biosensors have been prepared by adsorption of urease and butyrylcholinesterase on the heat-treated zeolite Beta crystals entrapped into the membranes deposited on the ion-selective field-effect transistor (ISFET) surfaces [13]. The zeolite-modified carbon paste electrode for simultaneous determination of dopamine and tryptophan has been described [14]. The usage of zeolites for fabrication of the biosensor for H_2O_2 detection based on cytochrome c was also considered [5].

The authors designed an Ag/NaA zeolite modified carbon paste electrode for DNA determination, which appeared to be very promising approach to further zeolites usage for this purpose [15].

The glucose biosensors prepared with zeolites incorporated into their bioselective membranes are also described in literature [16, 17].

Some advantages of silicalite application for the biosensor fabrication have been shown previously. It was demonstrated that the characteristics of conductometric urea biosensors based on urease adsorbed on silicalite are better than those of the biosensors based on urease immobilized in glutaraldehyde vapor [18]. Notably, the method of urease adsorption on silicalite is simple and rapid, it does not involve any toxic reagents.

The characteristics of stainless steel electrodes are quite perspective for the development of conductometric biosensors. In comparison to the electrodes based on other materials, such as platinum, gold, nickel, the stainless steel electrodes have been found to be high-sensitive to conductance changes, have a wide linear range of salt concentration detection, frequency stability, and a bit lower cost of production. However, these electrodes demonstrate poor enzyme adsorption on their surface, which complicates their utilization as transducers for the biosensors [19]. To enhance the adsorption, we have used silicalite since it is characterized by good adsorption properties, hydrophobic and organophilic selectivity, high thermal and chemical stability [20]. Therefore, the application of silicalite for improvement of the enzyme adsorption on the stainless steel electrodes was the main object of this work.

Materials and methods. *Materials*. Enzyme glucose oxidase (GOx), activity of 130 U/mg, from *Penicillium vitale* (EC 1.1.3.4) was obtained from «Diagnosticum» (Ukraine); glycerol, bovine serum albumin (BSA, fraction V), 50 % aqueous solution of glutaraldehyde (GA), and glucose were purchased from «Sigma-Aldrich Chemie» (Germany).

Synthesis of silicalite. Silicalite was synthesized in the Middle-East Technical University (Turkey). The optimized molar composition of the gel used for syhthesis of Silicalite-1 is 1TPAOH:4TEOS:350H_2O. Tetraethylorthosilicate (TEOS, 95 %) was used as the silica source. Tetrapropylammoniumhydroxide (TPAOH, 25 %) was used as a template. By hydrolyzing tetraethoxysilane (TEOS) with tetrapropylammonium hydroxide (TPAOH) solution, a clear homogeneous solution was obtained under 6-h stirring at room temperature. Afterwards the resulting gel was placed in oven for 18 h at

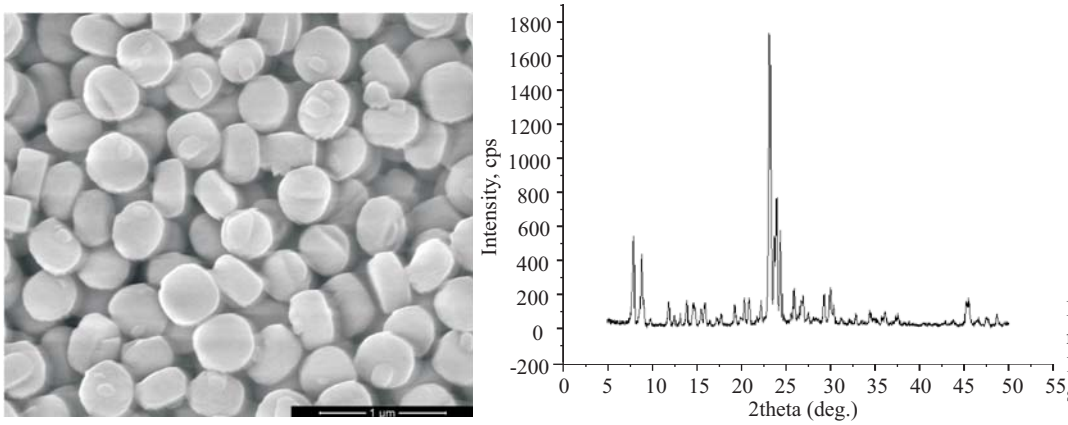

Fig. 1. Scanning electron microscopy image of silicalite (*A*), XRD spectrum of silicalite (*B*)

125 °C. To remove the unreacted material, the crystallized solid particles were centrifuged at 13000 rpm, washed with deionized water and dried at 80 °C. The SEM (scanning electron microscope) image of synthesized silicalite depicted in Fig. 1 shows that the prepared silicalite particles have size of about 400–500 nm.

Conductometric transducers. The conductometric transducers were 5×30 mm^2 in size and consisted of two identical pairs of stainless steel interdigitated electrodes deposited onto a ceramic support by successive thermo vacuum sputtering of titanium (adhesion layer) and stainless steel. Usage of two electrode pairs enabled a differential mode of measurements.

The sensitive area of each electrode pair was about 1.5×2 mm^2. The digits as well as inter-digital spaces were 50 μm wide each.

Preparation of silicalite modified electrode (SME). A silicalite layer on the transducer surface was formed by drop-coating. We used 10 % (w/w) silicalite solution in 5 mM phosphate buffer, pH 7.0. A constant amount (0.165 ml) of silicalite solution was deposited in the active zone of each pair of electrodes, and then the transducer was heated for 2 min at 200 °C. This temperature had no effect on the transducer working parameters. The procedure resulted in the formation of silicalite layer in the electrodes active zones.

Preparation of bioselective membrane. To form a bioselective membrane on the electrodes, three methods of glucose oxidase immobilization were used (Fig. 2, *A*). Equal amounts of enzyme were immobilized on the electrodes according to all these methods to provide an assessment consistency. The schematic view of the conductometric enzyme biosensor based on the stainless steel electrodes is presented in Fig. 2, *B*. The enzymatic reaction underlying a quantitative glucose determination by conductometric biosensors is presented in Fig. 2, *C*.

Glucose oxidase adsorption on silicalite modified electrodes (GOx-SME). To prepare a bioselective membrane we used the transducer previously coated with silicalite (see above). Then a constant amount (0.15 ml) of 5 % (w/w) GOx in 20 mM phosphate buffer solution, pH 7.0, was deposited onto one pair of electrodes whereas the same amount of 5 % (w/w) BSA in analogous buffer solution – onto the reference pair of electrodes; then the transducer underwent drying for 15 min at room temperature.

Neither GA nor other auxiliary compounds were used; GOx was immobilized onto the silicalite surface by physical adsorption. Next, the transducers were submerged into the working buffer solution for 10–15 min to wash off the unbound enzyme before the measurements.

Glucose oxidase immobilization in GA drop (GOx-GA). To prepare the enzyme membrane, the solution containing 10 % (w/w) GOx, 10% (w/w) BSA, 20% (w/w) glycerol in 20 mM phosphate buffer, pH 7.0, was used. The mixture for reference membrane was prepared in analogous manner, except that GOx was replaced with BSA. Thus, the reference solution contained 20 % (w/w) BSA. Both solutions were separately mixed with 2 % aqueous solution of glutaraldehyde in a ratio of 1:1. Immediately afterwards the mixture of enzyme solution with GA was deposited on one pair of electrodes and the mixture of reference solution with GA – on another. Time of immobilization was 30 min; glutaraldehyde formed strong covalent bonds between the compounds of bioselective membrane, whereas the bioselective membrane as a whole was attached to the electrode surface through weak (*i. e.*, Van der

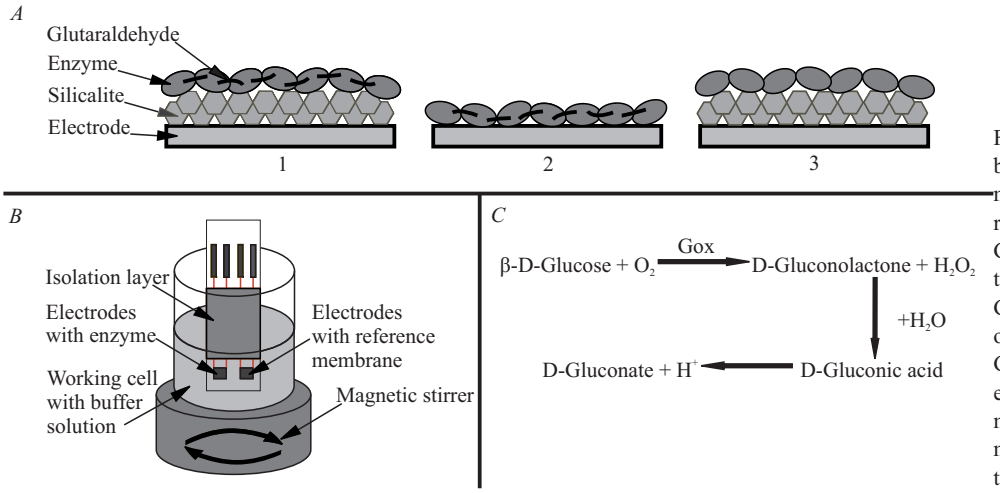

Fig. 2. Preparation and function of biosensor: *A* – glucose oxidase immobilization on stainless steel electrodes (*1* – GOx cross-linking with GA along with adsorption on silicalite modified electrodes (GOx-SME-GA); *2* – GOx cross-linking with GA on bare electrodes (GOx-GA); *3* – GOx adsorption on silicalite modified electrodes (GOx-SME)); *B* – schematic view of conductometric enzyme biosensor; *C* – enzymatic reaction in bioselective membrane

Waals) bonds. After immobilization, the electrodes were submerged in the working buffer for 30 min to washout the unbound enzyme and GA excess.

Glucose oxidase immobilization in GA drop on silicalite modified electrodes (GOx-SME-GA). The third method of immobilization was a combination of two previous methods. We modified the electrode surface with silicalite (see above) and then deposited the mixture of enzyme solution with GA on one pair of electrodes and the mixture of BSA solution with GA – on another (see above).

Measurement procedure. Measurements were carried out at room temperature in 5 mM phosphate buffer solution, pH 7.0, continuously stirred in an open 2 ml cell. The substrate concentrations in the cell were varied by addition of different aliquots of the stock solution. All experiments were repeated in triplicate. The data in the figures were presented either as a mean of three repeated results of the experiment or as a mean ± ± standard deviation (SD). The nonspecific changes in the output signal induced by fluctuations of temperature, medium pH, *etc*. were avoided due to the usage of differential mode of measurement.

Electrochemical measuring system. The conductometric determination of glucose, using the prepared biosensors, was realized in a differential measuring mode, which ensured satisfactory detection accuracy and suppression of non-informative effects of the environment (variations of temperature, pH and background conductivity of working solution).

The portable measuring device (9.5 × 2.5 × 13.5 cm) was produced at the Institute of Electrodynamics, NAS of Ukraine (Kyiv, Ukraine). The applied sinusoidal potential with frequency of 36.5 kHz and amplitude of 14 mV allowed avoiding such effects as Faraday processes, double-layer charging and polarization of the microelectrodes. Illumination and temperature variations had practically no influence on the biosensor characteristics. The measurements were carried out in a glass cell filled with phosphate buffer (volume 2 ml), under vigorous magnetic stirring.

Results and discussion. *Comparison of three methods of glucose oxidase immobilization*. Three methods of enzyme immobilization on the surface of conductometric transducers were compared in terms of the biosensor sensitivity (Fig. 3).

The GOx-SME-GA biosensors demonstrated the highest responses. Their values corresponded to the amount of enzyme immobilized on the transducers surface. Thus, it can be presumed that the largest amount of GOx was immobilized by simultaneous application of both procedures, *i. e*. adsorption on silicalite and cross-linking via GA; silicalite itself did not adsorbed a lot of GOx, and in case of cross-linking via GA it was difficult to wash out the immobilized enzyme because of weak interaction between stainless steel electrodes and cross-linked enzymes.

A main disadvantage of the biosensors with GOx adsorbed on silicalite without GA was gradual washout of GOx from the electrode surface covered with silicalite into working solution due to weak bounds between GOx and silicalite. Nevertheless, a combination of adsorption on silicalite and cross-linking by GA demonstrated very good results.

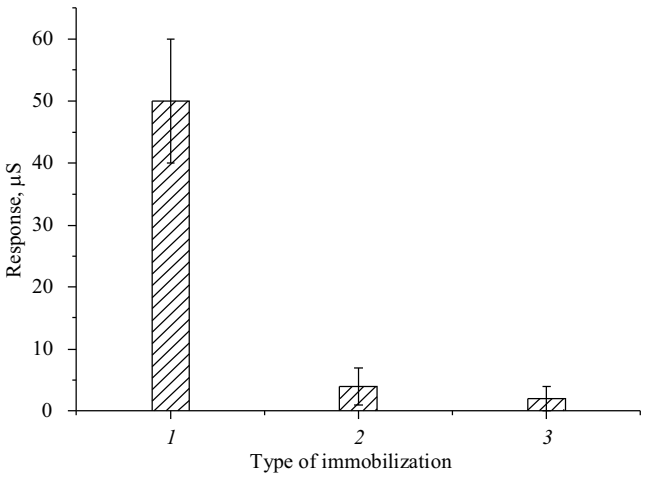

Fig. 3. Responses to 0.2 mM glucose of biosensors with different types of GOx immobilization: *1* – GOx-SME-GA; *2* – GOx-GA; *3* – GOx-SME. Measurements were carried out in 5 mM phosphate buffer, pH 7.0

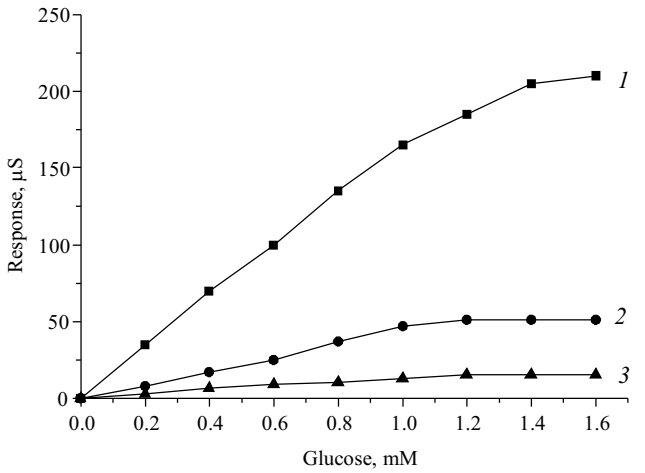

Fig. 4. Calibration curves of glucose conductometric biosensors with different types of GOx immobilization: *1* – GOx-SME-GA; *2* – GOx-GA; *3* – GOx-SME. Measurements were carried out in 5 mM phosphate buffer, pH 7.0

We also studied the linear range of operation. The calibration curves for glucose determination by the biosensors created with different methods of GOx immobilization are presented in Fig. 4. As seen, the linear range for GOx-SME-GA biosensors was 0–1.4 mM, for GOx-SME and GOx-GA biosensors 0–1.0 mM.

Thus, the method of GOx immobilization with GA on silicalite showed the most appropriate parameters for the creation of biosensors.

Reproducibility of biosensors construction and responses reproducibility. Reproducibility of biosensors construction is important for their standardization; therefore, this parameter was checked for three groups of biosensors. From the data presented in Fig. 5, *A*, it was calculated that the error of reproducibility of biosensors construction (relative standard deviation – RSD) for the GOx-SME-GA biosensors was 18 %, for GOx-GA biosensors – 76 % and for GOx-SME biosensors – 65 %.

Only the GOx-SME-GA biosensors were used in further studies since they showed the highest sensitivity and reproducibility of their construction. To determine reproducibility of responses, the biosensors responses to 0.2 mM glucose were measured within one working day with 10–15-min intervals; between measurements the biosensors were kept in the continuously stirred buffer solution. An error (RSD) of glucose measurements was 7 %, which is quite acceptable (Fig. 5, *B*).

Storage stability of GOx-SME-GA biosensors. An important stage in our work was the investigation on stability of the developed GOx-SME-GA biosensor during several days. The biosensor signal to 0.2 mM glucose was measured several times during 19 days with certain intervals. Between measurements the biosensors were stored dry at 4–8 °C. The results are presented in Fig. 6. After 18 days the responses decreased to 71 % of initial value, which is better than typical stability of the biosensors based on adsorbed enzymes. Thus, this method of immobilization gives the opportunity of long-term use of biosensors.

In general, the method of immobilization with GA and silicalite improved working characteristics of the biosensors based on stainless steel transducers in comparison with other methods described here. According to the results obtained, the GOx-SME-GA method of immobilization is rather perspective. This method can be used in future for immobilization of complex enzyme system or some unstable enzymes.

Conclusions. The methods of enzyme adsorption on the silicalite-modified electrode using GA (GOx-SME-GA) and without GA (GOx-SME) were compared with the traditional method of enzyme immobilization by cross-linking via glutaraldehyde without silicalite (GOx-GA). The GOx-SME-GA biosensors with stainless steel electrodes were characterized by 12–25 times higher sensitivity compared with the biosensors construction two other methods of GOx immobilization. The GOx-SME-GA biosensors demonstrated the storage stability with only 29 % loss of activity after 18 days, the satis-

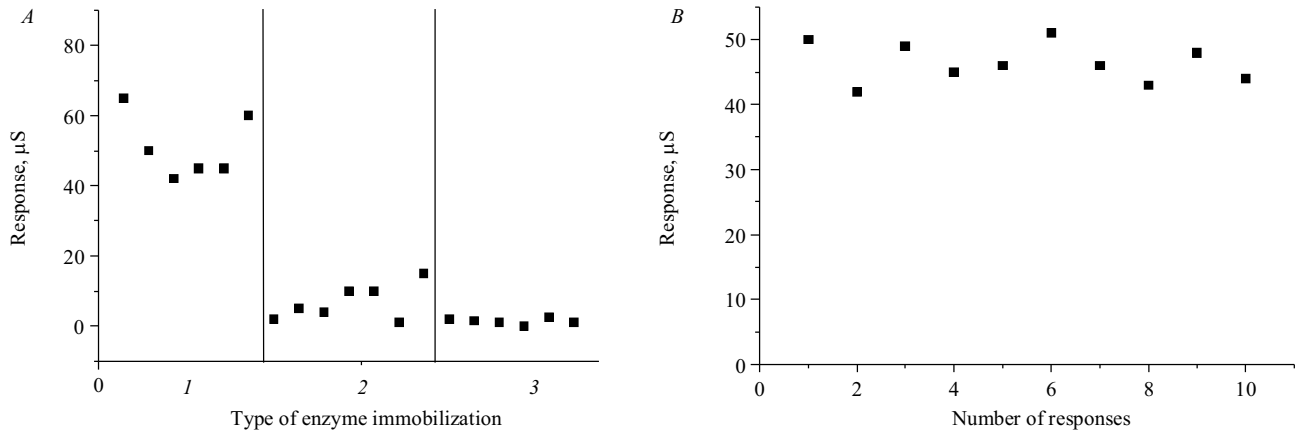

Fig. 5. *A* – reproducibility of glucose biosensors construction based on stainless steel electrodes with different types of GOx immobilization (*1* – GOx-SME-GA; *2* – GOx-GA; *3* – GOx-SME); *B* – signal reproducibility of GOx-SME-GA biosensor. Measurements were carried out in 5 mM phosphate buffer, pH 7.0, glucose concentration was 0.2 mM (*A*, *B*)

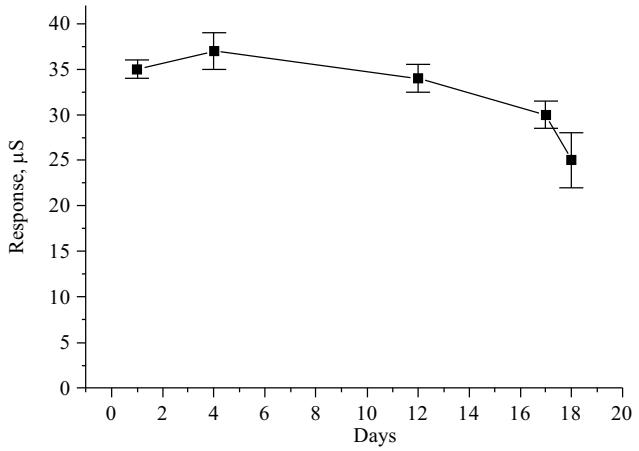

Fig. 6. Storage stability of GOx-SME-GA biosensor. Measurements were carried out in 5 mM phosphate buffer, pH 7.0, glucose concentration was 0.2 mM

factory reproducibility of biosensor construction (RSD – 18 %), good response reproducibility (RSD of glucose determination – 7 %). These data permit to state that the complex use of GA and silicalite sufficiently enhances the enzyme adsorption on the stainless steel electrodes. Thus, the method of enzyme immobilization using silicalite along with GA is highly effective for the creation of a sensitive biosensor with good signal reproducibility.

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Використання силікаліту для покращення адсорбції ферменту на поверхні перетворювачів з нержавіючої сталі

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Резюме

Мета. *Покращення аналітичних характеристик ферментних біосенсорів на основі нових недорогих перспективних електродів з нержавіючої сталі за допомогою наночастинок силікаліту.* ***Методи***. *Використано кондуктометричний біосенсор з іммобілізованою глюкозооксидазою як біоселективним елементом та сталеві електроди як перетворювачі.* ***Результати***. *Застосовано і порівняно три методи іммобілізації глюкозооксидази (ГО) на поверхні датчиків: адсорбція ГО на модифікованій частинками силікаліту поверхні електрода; поперечне зшивання ГО з глутаровим альдегідом (ГА) без використання силікаліту; сорбція ГО на модифікованому силікалітом електроді у комбінації з поперечним зшиванням з ГА. Біосенсори з ферментами, іммобілізованими на поверхні сталевого електроду за рахунок сорбції на шарі силікаліту у комбінації з поперечним зшиванням з ГА, мають в 12–25 разів вищу чутливість порівняно з іншими біосенсорами. Ця ж група біосенсорів характеризується високою відтворюваністю сигналів між різними партіями (відносне стандартне відхилення (ВСВ) становить 18 %), а також відтворюваністю в одній партії з ВСВ 7 %. Таким біосенсорам притаманна висока стабільність при зберіганні (втрата лише 29 % від первинного сигналу після 18 днів зберігання).* ***Висновки***. *Показано, що використання частинок силікаліту поряд з методом поперечного зшивання з ГА значно підвищує сорбцію ферментів на поверхні датчиків з нержавіючої сталі під час іммобілізації, а також покращує аналітичні параметри біосенсорів. Цей метод іммобілізації ферментів може бути застосований для подальшого удосконалення роботи біосенсорів.*

Ключові слова: іммобілізація ферментів, силікаліт, глюкозооксидаза, кондуктометричний перетворювач, біосенсор.

Использование силикалита для улучшения адсорбции фермента на поверхности преобразователей из нержавеющей стали

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Резюме

Цель. *Улучшение аналитических характеристик ферментных биосенсоров на основе новых недорогих перспективных электродов с помощью наночастиц силикалита.* ***Методы***. *Использовали кондуктометрический биосенсор с иммобилизованной глюкозооксидазой в качестве биоселективного элемента и стальные электроды как преобразователь.* ***Результаты***. *Сопоставлены между собой три метода иммобилизации глюкозооксидазы (ГО) на поверхности преобразователей: адсорбция ГО на поверхности модифицированных силикалитом электродов; поперечная сшивка ГО с глутаровым альдегидом (ГА) без использования силикалита; адсорбция ГО на модифицированном силикалитом преобразователе в комбинации с поперечной сшивкой с ГА. Биосенсоры, созданные вследствие комбинации сорбции ГО на слое силикалита на поверхности стального электрода и сшивки с ГА, имеют чувствительность в 12–25 раз выше, нежели другие биосенсоры. Биосенсоры этой же группы отличаются высокой воспроизводимостью сигналов между разными партиями (относительное стандартное отклонение (ОСО) составляет 18 %), и воспроизводимостью внутри одной партии с ОСО 7 %. Такие биосенсоры обладают высокой стабильностью при хранении (потеря чувствительности в первые 18 дней хранения достигает лишь 29 %).* ***Выводы***. *Показано, что использование частиц силикалита одновременно с методом поперечной сшивки с ГА в значительной степени повышает сорбцию ферментов на поверхности преобразователей из нержавеющей стали во время иммобилизации, а также улучшает аналитические парметры бисоенсоров. Такой метод иммобилизации ферментов может быть применен для дальнейшего усовершенствования работы биосенсоров.*

Ключевые слова: иммобилизация ферментов, силикалит, глюкозооксидаза, кондуктометрический преобразователь, биосенсор.

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