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BIOSENSORS

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POTENTIALITIES OF ZEOLITES FOR IMMOBILIZATION OF ENZYMES IN CONDUCTOMETRIC BIOSENSORS

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Abstract. Conductometric biosensors based on urease and glucose oxidase immobilized with different types of zeolites have been investigated and compared. For this purpose, zeolite A, zeolite Y, Silicalite-1 (spherical), Silicalite-2, H+Beta 300, H+Beta 150 and NH₄+Beta 25 were compared as potential carriers for enzyme immobilization. The parameters to obtain optimized biosensor performance were studied by investigating the percentage of zeolite in membrane, immobilization time in glutaraldehyde vapor and pH of the environment. Different zeolite types resulted in different enzymatic responses. In particular, we have demonstrated that the urease immobilized on silicalite-2 had better performance than immobilized urease without zeolite. Conductometric biosensor with glucose oxidase immobilized with NH₄+Beta 25 zeolite had similar response values compared with immobilised enzyme without zeolite. The results obtained show that zeolites could be used as alternatives for enzyme immobilization in conductometric biosensors development.

Keywords: conductometric biosensor, urease, glucose oxidase, zeolite, silicalite

ПОТЕНЦІЙНІСТЬ ВИКОРИСТАННЯ ЦЕОЛІТІВ ДЛЯ ІММОБІЛІЗАЦІЇ ФЕРМЕНТІВ В КОНДУКТOMETРИЧНИХ БІОСЕНСОРАХ

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Анотація. В роботі досліджено та порівняно кондуктометричні біосенсори на основі ферментів уреазы та глюкозооксидазы, іммобілізованих з різними типами цеолітів. Для цього з метою вивчення потенціалу їх використання як основи для іммобілізації ферментів було порівняно наступні матеріали: цеоліт А, цеоліт Y, сілікаліт-1 (сферичний), сілікаліт-2, H+Beta 300, H+Beta 150 та NH₄+Beta 25. Було вивчено параметри для отримання оптимальних характеристик біосенсорів, а саме процент цеолітів в мембрані, час іммобілізації в парах глутарового альдегіда та рН середовища. Отримано результати по впливу різних цеолітів на різні ферментативні реакції, наприклад було показано, що уреазы, іммобілізована з сілікалітом-2, демонструвала кращі характеристики за уреазы без цеоліту. Кондуктометричний біосенсор з глюкозооксидазою, іммобілізованою з цеолітом NH₄+Beta 25 мав подібні величини відгуків у порівнянні з іммобілізованим ферментом без цеоліту. З отриманих результатів можна зробити висновок, що цеоліти можуть бути використані як альтернативні носії для іммобілізації ферментів при розробці кондуктометричних біосенсорів.

Ключові слова: кондуктометричний біосенсор, уреазы, глюкозооксидаза, цеоліт, сілікаліт

ВОЗМОЖНОСТЬ ПРИМЕНЕНИЯ ЦЕОЛИТОВ ДЛЯ ИММОБИЛИЗАЦИИ ФЕРМЕНТОВ В КОНДУКТOMETРИЧЕСКИХ БИОСЕНСОРАХ

Есін Сої, Вікторія Пешкова, Валентина Архипова, Басма Хадро, Ніколь Джаффрезік-Рено, Альберт Сакко, Сергій Дзядевич, Бурджу Аката

Аннотация. В работе исследованы и сравнены кондуктометрические биосенсоры на основе ферментов уреазы и глюкозооксидазы, иммобилизованные с различными типами цеолитов. Для этого с целью изучения возможностей их применения в качестве матрицы для иммобилизации ферментов сравнивались следующие материалы: цеолит А, цеолит Y, силикалит-1 (сферический), силикалит-2, H+Beta 300, H+Beta 150 и NH₄+Beta 25. Были изучены параметры для получения оптимальных характеристик биосенсоров, а именно процент цеолитов в мембране, время иммобилизации в парах глутарового альдегида и рН среды. Были получены результаты по влиянию разных цеолитов на различные ферментативные реакции, например, показано, что уреазы, иммобилизованная с силикалитом-2, демонстрирует лучшие характеристики чем уреазы без цеолита. Кондуктометрический биосенсор с глюкозооксидазой, иммобилизованной с цеолитом NH₄+Beta 25 имел подобные величины откликов по сравнению с иммобилизованным ферментом без цеолита. Из полученных результатов можно сделать вывод, что цеолиты могут быть использованы в качестве альтернативных носителей ферментов при разработке кондуктометрических биосенсоров.

Ключевые слова: кондуктометрический биосенсор, уреазы, глюкозооксидаза, цеолит, силикалит

1. Introduction

Research and development in biosensors have gained increasing importance in the last few years for their advantageous properties as analytical tools, namely the simplicity of use, potential miniaturization, portability and low cost, in comparison with well-established lab-based methods. Nowadays it

is well established that the performance of biosensors depends greatly on the influence imposed on biomolecules by the immobilization technique. Immobilization is the key-step in biosensor construction, but, regardless their peculiar advantages, the conventional methods for biomolecule immobilization (physical adsorption, covalent binding, cross-

linkings and entrapment in gels or membranes) have, in general, low reproducibility and poor spatially controlled deposition, a crucial problem for the development of commercial miniaturized biosensors [1]. In this context, the use of nanomaterials for the construction of biosensing devices constitutes one of the most exciting approaches. The extremely promising prospects of these devices accrue from the unique properties of nanomaterials, i.e., high surface area, local chemical environment, tailorable surface groups.

Application of nanomaterials in biosensors allows the use of many new signal transduction technologies in their manufacture. Nanomaterials can be used in a variety of electrochemical biosensing schemes thereby enhancing the performance of these devices and opening new horizons in their applications.

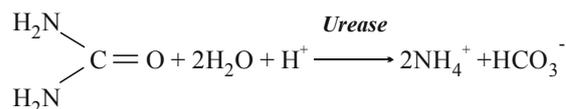
The success of immobilization of enzymes depends strongly on the properties of the carriers employed. The carrier material should have a high capacity to bind enzyme, should be mechanically stable and must not have reduced the enzymatic activity. The organic supports like polymers lead to a number of problems such as poor stability and disposal issues [2]. In contrast, inorganic materials such as silica and alumina are thermally and mechanically stable and strong [3, 4].

Among different alternatives for carriers, zeolites have been showing great promise as carriers, due to their increased surface area, tunable hydrophilic/hydrophobic properties and Bronsted acidity by controlling the Si/Al ratio as well as their thermal and mechanical stabilities [5-10]. In this way higher stability and increased activity of the enzyme can be obtained. Accordingly, zeolite Y and silicalite are the commonly used potential carriers for immobilization of different types of proteins like lipase [11, 12], albumin [13], and trypsin [14] in biotechnological processes. In such studies, which used zeolites as potential carriers for enzyme immobilizations, higher stability, reusability, and productivity were observed.

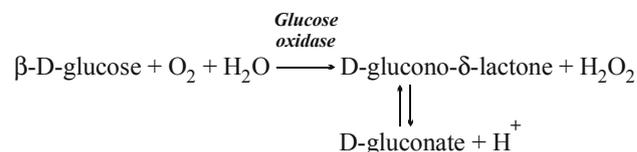
Conductometric biosensors are based on the fact that almost all enzymatic reactions involve either consumption or production of charged species and, therefore, lead to a global change in the ionic composition of the tested sample [15]. Biosensors based on the conductometric principle present a number of advantages: a) thin-film electrodes are suitable for miniaturisation and large scale production using inexpensive technology, b) they do not require

any reference electrode, c) transducers are not light sensitive, d) the driving voltage can be sufficiently low to decrease significantly the power consumption, e) large spectrum of compounds of different nature can be determined on the basis of various reactions and mechanisms.

In the case of the urea assay, resulting conductivity changes are produced by enzymatically catalyzed hydrolysis of the substrate:



while for glucose these are due to the dissociation of gluconic acid produced as a result of the enzymatic oxidation of glucose according to the scheme:



In the current study, the use of zeolites for enzyme immobilization in conductometric biosensors based on urease and glucose oxidase was tested for the first time.

2. Materials and methods

2.1. Materials. The frozen-dried preparations of enzymes used in the experiments were as follows: glucose oxidase (GOD) from *Penicillium vitale* (EC 1.1.3.4) with activity of 130 U/mg from *Diagnosticum* (L'viv, Ukraine); urease from soybeans (EC 3.5.1.5, type B) with activity of 22 U/mg from *Sigma-Aldrich Chemie*. Bovine serum albumin (BSA) (V fraction) and 50 % aqueous solution of glutaraldehyde (GA) were obtained from *Sigma-Aldrich Chemie*. Glucose and urea were used as a substrate and analyzed substance, potassium-phosphate solution ($\text{KH}_2\text{PO}_4\text{-NaOH}$), pH 7.2 from *Merck* was used as a buffer. Other non-organic compounds were of analytical grade.

Commercial samples of zeolite Y, zeolite $\text{NH}_4\text{-Beta-25}$ (Si/Al ratio of 12.5), H-Beta-150 (Si/Al ratio of 75) and H-Beta-300 (Si/Al ratio of 150) were obtained from Sud-Chemie (USA). Zeolite A (cubic) and two silicalite samples with two different particle sizes (Silicalite-1 is around 800 nm and Silicalite-2 is around 350-400 nm) were synthesized in Middle East Technical University, Micro and Nanotechnology Department (Turkey).

2.2. Synthesis of zeolites and silicalite. Zeolite and silicalite particles were hydrothermally synthesized according to Table 1 and detailed synthetic conditions and reagents are listed. All the reagents were chemically or analytically pure and

used without any purification. The particle size and Si/Al ratios of zeolite crystals were determined using scanning electron microscope (SEM) and energy dispersive X-ray spectrometer (EDS) and listed in Table 2.

Table 1

List of zeolite samples and their synthesis procedures

Zeolite Type	Chemical Composition of Gel	Temperature (°C)	Crystallization Time (h)
Zeolite A	11.25SiO ₂ :1.8Al ₂ O ₃ :13.4(TMA) ₂ O:0.6Na ₂ O:700H ₂ O.	60	24
Zeolite Y	Commercial	-	-
Silicalite 2	1TPAOH:4TEOS:350 H ₂ O	125	18
Silicalite 1	1TPAOH:5TEOS:500H ₂ O	175	6
H+Beta 300	Commercial	-	-
H+Beta 150	Commercial	-	-
NH ₄ +Beta 25	Commercial	-	-

Table 2
Particle size, surface area and Si/Al ratio of synthesized zeolites

	Particle Size	Pore Size* (E)	Si/Al Ratio
Zeolite A	~250 nm	0.41	~ 1,35
Zeolite Y	1.5-2 μm	0.74	~ 2,39
Silicalite 2	~400 nm	0.53 x 0.56	No Al
Silicalite 1	~800 nm	0.53 x 0.56	No Al
H+Beta 300	1-2 μm	7.6 x 6.4	~150
H+Beta 150	1.5-3 μm	7.6 x 6.4	~75
NH ₄ +Beta 25	1-3 μm	7.6 x 6.4	~12.5

*Taken from <http://www.iza-structure.org/databases/>

The morphology of particles of investigated zeolites are shown in Fig. 1. According to their SEM images as seen in Figures 1 and X-ray diffractions (XRD-not shown), all of the samples were well crystallized.

2.3 Sensor design. The conductometric transducers were produced according to our recommendations in Lashkarev Institute of Semiconductor Physics of National Academy of Sciences of Ukraine (Kyiv, Ukraine). They were consisted of two identical pairs of gold interdigitated electrodes made by gold vacuum evaporation onto pyroceramic substrate (5 x 40 mm). The surface of sensitive area of each electrode pair was about 1.0 x 1.5 mm. The width of each of interdigital spaces and digits was 20 μm.

2.4 Bioselective membrane production. The solution consisting of enzyme in 20 mM phosphate buffer, pH 7.2, with 10% glycerol was used to produce the working membrane, while the same mixture

with BSA instead of enzymes was used for the reference membrane. Immobilization was carried out after deposition of 0.5 μL of each solution on each electrode pair and then exposure to GA vapor. The protein content was the same in both membranes. Before usage, the sensors were dried in the air at ambient temperature for 10 min and then were exposed to the working buffer solution.

In the case of enzyme immobilization with zeolites, the zeolites were prepared and added to the immobilization mixture with different concentrations.

2.5 The measurement procedure. The measurements were carried out in an open cell at room temperature. The 10 mM phosphate buffer or universal buffer at different pH values were intensively stirred. The necessary substrate concentration in the working buffer was achieved by adding given portions of the stock substrate solution. The experiments were repeated at least three times sequentially. The effect of nonspecific variations of output signal owing to temperature and pH changes and electric interferences was avoided by operating in the differential mode.

3. Results and discussion

3.1 Response time of the biosensors

A typical dependence of the conductometric biosensor response on the time after glucose additions is shown in Fig. 2. After the biosensor reached a stable baseline in blank phosphate buffer solution, injection of glucose stock solutions caused significant sensor response, which resulted from subsequent

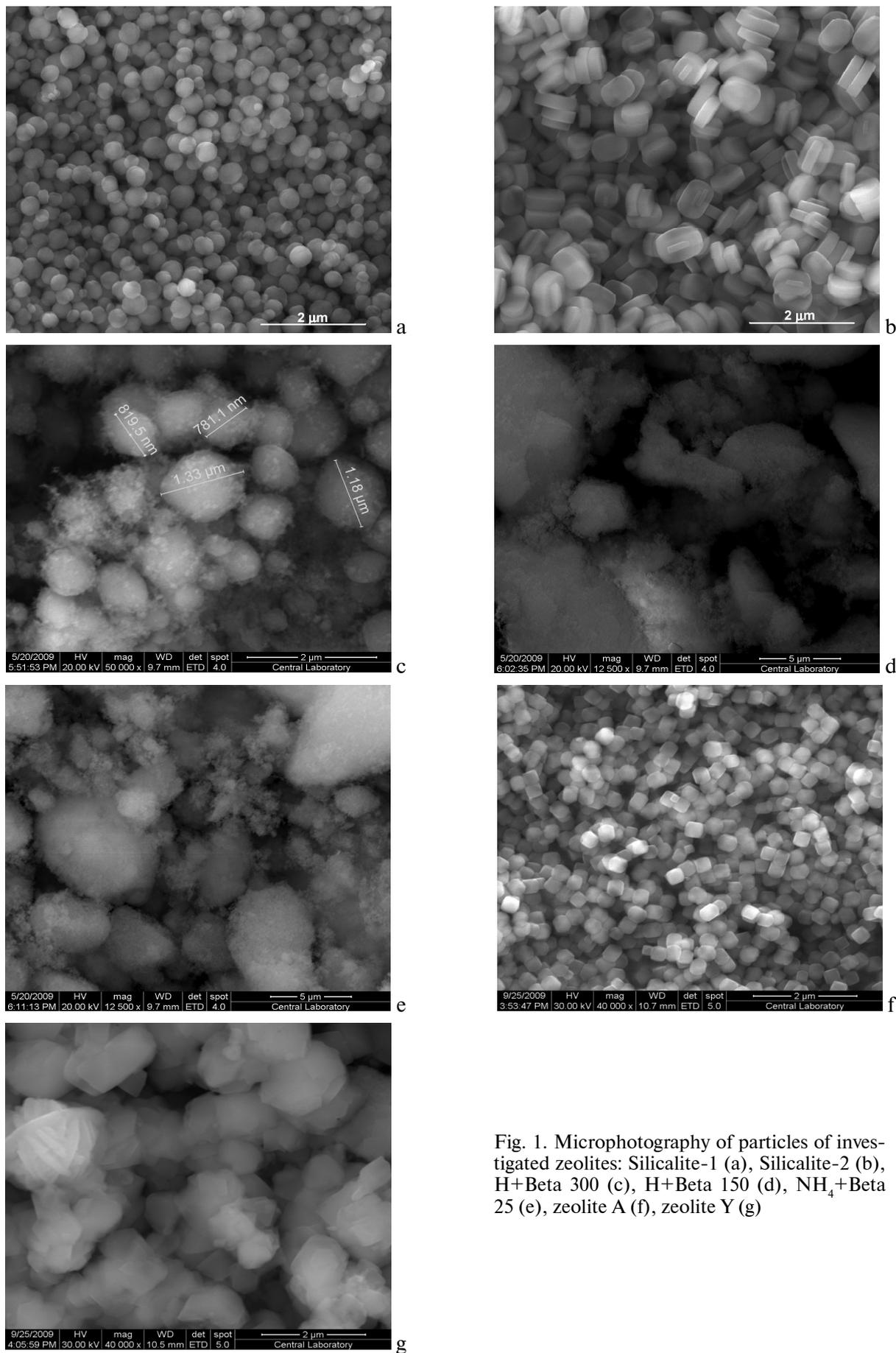


Fig. 1. Microphotography of particles of investigated zeolites: Silicalite-1 (a), Silicalite-2 (b), H+Beta 300 (c), H+Beta 150 (d), NH₄+Beta 25 (e), zeolite A (f), zeolite Y (g)

local increasing of concentration of ionic species inside the membrane. As can be seen the biosensor steady-state response times i.e., times necessary to reach 90 % of the steady-state amplitude were about 1-2 min.

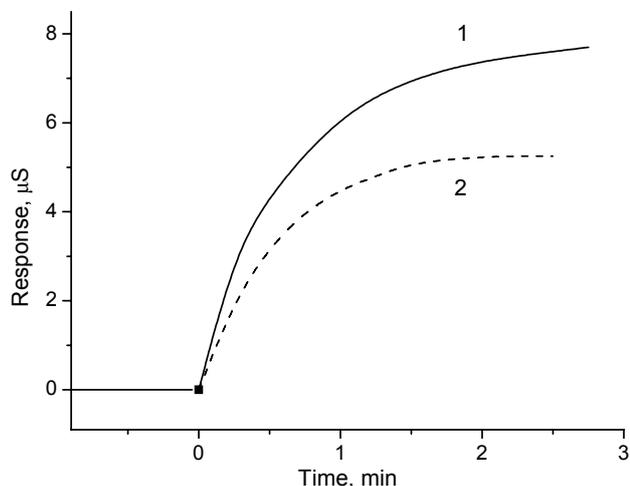


Fig. 2: A typical response curves of conductometric biosensor based on glucose oxidase using Silicalite-1 for 1 mM (1) and 0.6 mM (2) glucose. Measurements were conducted in 10 mM phosphate buffer, pH 7.4

3.2 Effect of zeolite loading. Initially the effect of the zeolite loading in immobilisation mixture on biosensor response to 0.2 mM glucose was investigated. Different concentrations of Silicalite-2 in the membrane were tested to optimise the amount of zeolite loading with the sensor response (Fig. 3). The responses of biosensors with 5% zeolites were low compared to responses with lower concentration of zeolites. The level of saturation of biosensors also differs depending on concentration of zeolites in the membranes. The biosensors utilizing 0.5% zeolite loadings were used for further experimental work.

3.3 Effect of exposure time to GA vapour. In order to determine the effect of cross-linking time for the enzyme, electrodes were kept in GA vapour for different times. As seen in Fig. 4 for zeolite A, a 25 minute exposure time in GA vapour was observed to lead to the highest response and thus the optimum time for cross-linking of urease. In fact, if the exposure time is kept short for immobilization, enzyme leakage through the membrane can take place because of insufficient bonding. This would lead to poor stability of the biosensors and the response of the sensors are observed to decrease accordingly. On the other hand, if the exposure time for immobilization is longer than the optimum value, a decrease of response was observed. This can be related to the formation of a large number of bonds

between glutaraldehyde and the enzyme molecules, which leads to strong membrane. Thus the active site of the enzyme can be inaccessible.

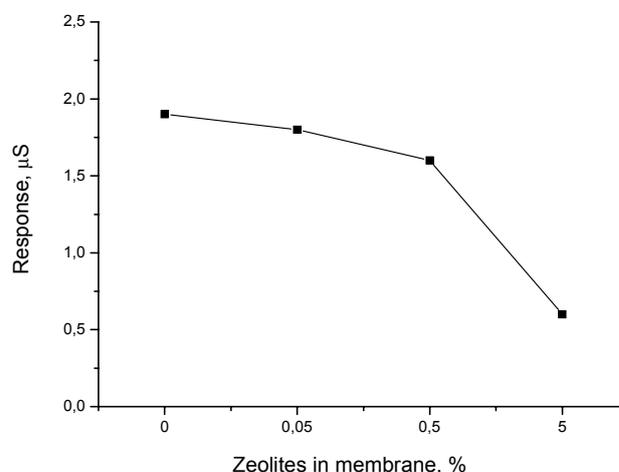


Fig. 3. The dependence of responses of glucose biosensors on weight percent of zeolite (Silicalite 2) in membranes. Membrane composition: 5% GOD, 5% BSA, 20 mM PBS, pH 7,2, 10% Glycerin

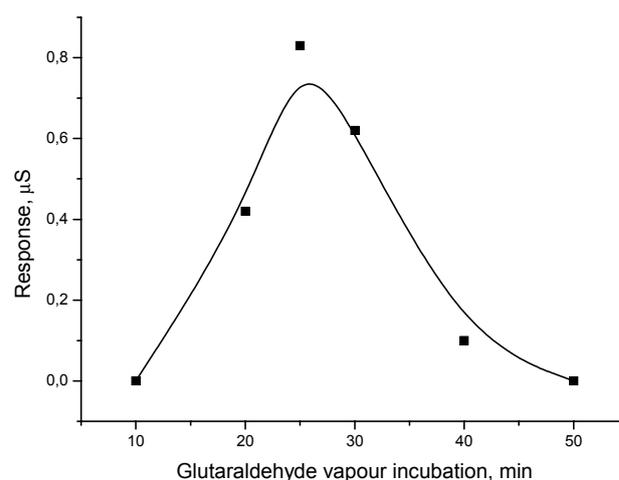


Fig. 4. The dependence of responses of conductometric biosensors on cross-linking time in glutaraldehyde vapour for urease and zeolite A in biosensor membranes

3.4 Effect of pH on biosensor response. It is well known that the choice of buffer may influence the enzyme activity. The most commonly used buffer in similar systems is phosphate solution. Fig. 5 shows the variation of the response at different pH values in the carrier solution. It can be seen that an optimum for the sensor response was observed using the solution with pH 7.5.

3.5 Effect of the zeolite nature on biosensor response. As seen on Fig. 6, urease with silicalite-2 showed the highest response compared to other zeolites and also higher than the urease immobi-

lized without zeolite. Silicalite-2 has no aluminum, and thus it is the most hydrophobic material among all samples investigated. Furthermore, zeolite Y showed slightly higher response than zeolite A. This may be related to the relatively higher pore size and Si/Al ratio and surface area of zeolite Y with respect to zeolite A crystals.

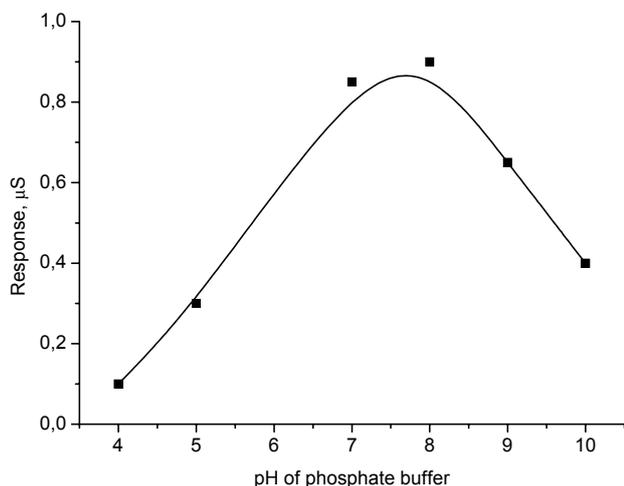


Fig. 5. The dependence of responses of conductometric biosensors on pH for urease and zeolite A in membranes

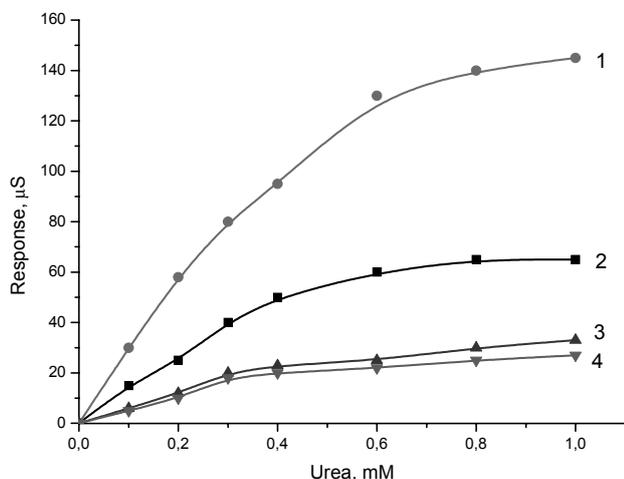


Fig. 6. Calibration curves for urea determination for urease immobilized with silicalite-2 (1), without zeolite (2), zeolite Y (3), and zeolite A (4)

The calibration curves of the laboratory prototypes of conductometric biosensors based on glucose oxidase without zeolite and with various zeolites are shown in Fig. 7. The linear dynamic range of glucose determination was until 1-1.5 mM in 10 mM phosphate buffer solution with the detection limit of 200 nM. Analysis of working characteristics of developed biosensors demonstrated linear response to glucose for enzyme immobilized with

zeolites in almost the same concentration range as glucose oxidase immobilized without zeolite. As can be seen, biosensor with GOD immobilized with NH_4 +Beta 25 demonstrates the best working characteristics: low detection limit, high level of response, good storage and operational stability.

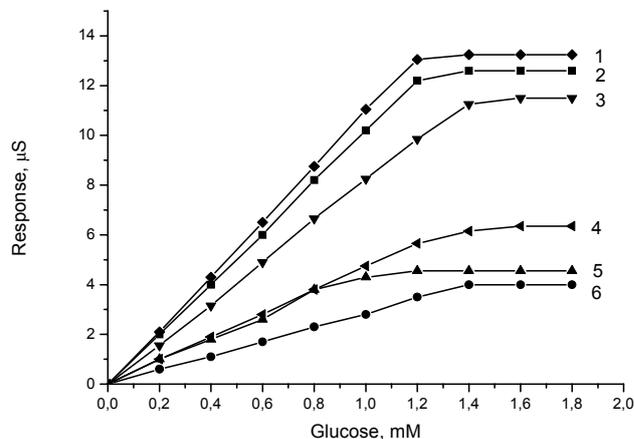


Fig. 7. Calibration curves for glucose determination for conductometric biosensor based on glucose oxidase immobilized with NH_4 +Beta 25 (2), Silicalite-1 (3), Silicalite-2 (4), H+Beta 300 (5), H+Beta 150 (6) and without zeolite (1)

4. Conclusion

This work describes a comparative study between the different parameters of conductometric biosensors based on urease and glucose oxidase immobilized with different types of zeolites. In particular, we have demonstrated that the urease immobilized on silicalite-2 had better performance than immobilized urease without zeolite. Conductometric biosensor with glucose oxidase immobilized with NH_4 +Beta 25 zeolite had similar response values compared to immobilised enzyme without zeolite. These results suggest that zeolites of different types can be used as alternatives for enzyme immobilization in conductometric biosensors development. It was shown that different zeolites with different characteristics lead to different biosensor results. Thus, it can be hypothesized that different properties of zeolites, such as their ion exchange behaviors, particle sizes, surface groups, pore sizes, and Si/Al ratios can be tailored in such a way that the optimum performance from a biosensor can be achieved upon choosing the right zeolite type and tuning its characteristic properties. Accordingly, our future research will focus on to evaluate such zeolite characteristics in detail for potential development of the optimum electrodes for desired purposes.

Acknowledgements

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