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A novel urea conductometric biosensor based on zeolite immobilized urease

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ABSTRACT

A new approach was developed for urea determination where a thin film of silicalite and zeolite Beta deposited onto gold electrodes of a conductometric biosensor was used to immobilize the enzyme. Biosensor responses, operational and storage stabilities were compared with results obtained from the standard membrane methods for the same measurements. For this purpose, different surface modification techniques, which are simply named as Zeolite Membrane Transducers (ZMTs) and Zeolite Coated Transducers (ZCTs) were compared with Standard Membrane Transducers (SMTs). Silicalite and zeolite Beta with Si/Al ratios 40, 50 and 60 were used to modify the conductometric electrodes and to study the biosensor responses as a function of changing zeolitic parameters. During the measurements using ZCT electrodes, there was no need for any cross-linker to immobilize urease, which allowed the direct evaluation of the effect of changing Si/Al ratio for the same type of zeolite on the biosensor responses for the first time. It was seen that silicalite and zeolite Beta added electrodes in all cases lead to increased responses with respect to SMTs. The responses obtained from ZCTs were always higher than ZMTs as well. The responses obtained from zeolite Beta modified ZMTs and ZCTs increased as a function of increasing Si/Al ratio, which might be due to the increased hydrophobicity and/or the acid strength of the medium.

1. Introduction

Enzyme-based biosensors have been of intense investigation [1–10]. The research has focused on enhancement of the sensitivity, detection limit, selectivity and the storage stability of these electrochemical biosensors. To be able to tailor these properties, variety of modification methods on electrochemical biosensor surfaces are proposed [2], since an important method in the development of enzyme-based biosensor is known to be the immobilization of enzymes on the transducer surfaces [3]. Electrode surfaces of the biosensors can be modified with different nanotechnology products such as sol–gels [4], nanotubes [5], polymers [6], and zeolites [2,7–10].

The use of zeolites in combination with enzymes have been of interest for a while due to some of their particular properties, such as tailorable surface groups, controlled hydrophilic/hydrophobic properties, shape, charge, and size selectivities, and their ability to regulate acidity for bi-functional enzymatic-acid catalysis. Furthermore, they are stable at high temperatures, insoluble in organic solvents, and resistant to harsh experimental conditions. Thus, they have been used to control the micro-environment of enzymes [11]. With such interesting properties, zeolites can offer themselves as alternative materials to be used for functionalizing solid substrates in a controlled manner. This can be of interest in the field of biosensors, and especially for conductometric ones, since they can be designed as integrated microbiosensors, which results into significantly reduced background conductivity due to the influence of temperature variations and other factors [12]. However, in order to use zeolites as alternative materials for enzyme immobilization and integrate them into such biosensor devices, the possibility to develop a simple and general technique to engineer the electrode surfaces for immobilization of biomolecules should be investigated. In this way, the potential advantageous roles for integrating zeolites can be explored by adsorbing enzymes on the surface of appropriate zeolites to obtain microdevices with high-sensitive biocatalytic function and long life biosensor property. The major challenge to use zeolites in such integrated devices and applications is its powder form upon synthesis. For using zeolites in these fields, zeolite films have to be constructed on the electrodes with controllable thickness and such electrodes are called as Zeolite-Modificated Electrodes (ZMEs). There have been different studies related with the construction of ZME's [13–15]; however

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studies reporting biosensor responses obtained from ZME's are scarce. Alain Walcarius made an attempt to classify the wide range of different fabrication procedures into 7 classes in his review [7]. These methodologies basically consist of adding/mixing zeolite particles with different composite materials, such as polymers and carbon paste and covalently tethering clay particles to the electrode surfaces. Furthermore, there has been no study on such systems in conductometric biosensors, which usually show certain limitations due to their signal to noise ratio. It is known that the need to use buffers for traditional conductometric applications in solution results in a drop in sensitivity, especially in the presence of non-reacting ions in the solution [12].

In our previous report, such a traditionally formed ZME was tested using conductometric electrochemical biosensors for the first time [16]. The advantage of such membranes is that there is no enzyme leakage from the membrane that results in a more stable electrode. However, it is time consuming and usage of another chemical makes the system more expensive. In literature, there are various reports about physical adsorption of urease on the transducer solid substrates with some supporting materials such as sol–gels [3,4], polymeric membranes [12,17], and microcapsules [18,19]. This approach can increase the adsorption capability of the solid substrate, but also reduce the catalytic activity [20] with an increased response time [4]. To our knowledge, the immobilization of urease on nanozeolite assembled electrodes for conductometric biosensors has not been explored.

In the present work, we introduce a new approach for urease determination where thin films of silicalite and zeolite Beta were obtained on the gold electrodes of conductometric biosensors. The responses, as well as the operational and storage stabilities were compared with the results obtained using traditional ZME's that were constructed for comparison purposes. The novelty of the current system with respect to the traditional ones is that there is no need for an extra membrane and the biosensor works as a result of the simple sorption of urease on the zeolite thin film. For that purpose, the modified electrodes were firstly developed by using silicalite samples and afterwards, zeolite Beta samples with different silicon to aluminum (Si/Al) ratios were used to modify the electrodes to compare the varying responses as a function of tailored electrode surfaces. The newly obtained ZME's that were constructed from the zeolite thin films on gold electrodes were compared with the "zeolite mixed" membrane transducers (ZMTs) and "un-modified" ones (SMTs). In this way, it was possible to investigate the effect of modification type on the biosensor responses using two different types of zeolites. Furthermore, Si/Al ratio was changed using the same type of zeolite, zeolite Beta, which allowed us to evaluate the effect of changing hydrophobicity of the transducer surfaces on the biosensor responses for the first time using conductometric biosensors.

The conductometric responses of such electrodes modified with zeolites were tested using the enzymatic reaction of urease:

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\text{urease}} 2NH_4^+ + HCO_3^- \quad (1)$$

This reaction results in a change of charged ions which results in local alteration of conductivity in the solution. This allows usage of conductometric electrodes as transducers. Accordingly, in the first part of this work, a conductometric biosensor was constructed using silicalite for the first time and different biosensor characteristics were compared with the membrane type electrode configurations, such as "Zeolite Membrane Transducers" (ZMTs) and "Standard Membrane Transducers" (SMTs), of conductometric electrodes that does and does not include silicalite particles, respectively.

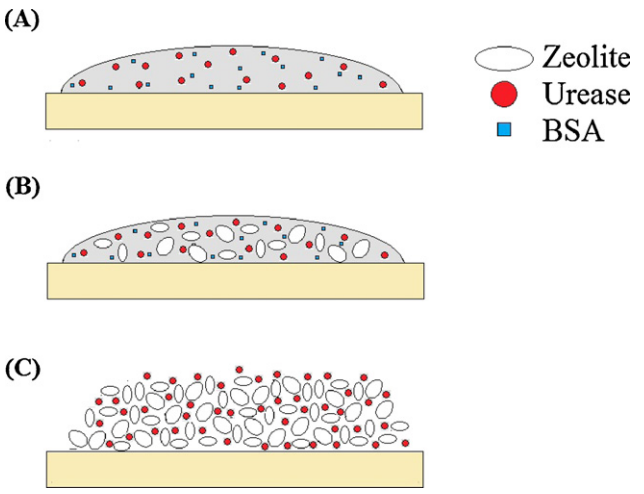

Fig. 1. Schematic representation of electrodes; (A) Standard Membrane Transducer (SMT), (B) Zeolite Membrane Transducer (ZMT) and (C) Zeolite Coated Transducer (ZCT).

2. Experimental

2.1. Materials

Crystals of zeolite Beta with Si/Al ratio of 40 and 50 synthesized with the molar formula of $1.92Na_2O{:}Al_2O_3{:}xSiO_2{:}4.6(TEA)_2O{:}444H_2O$ (where x is 40 and 50) and Si/Al ratio of 60 synthesized with the molar formula of $1.92Na_2O{:}0.5Al_2O_3{:}30SiO_2{:}4.6(TEA)_2O{:}444H_2O$ were prepared from two precursor solutions. A sodium aluminate solution was prepared by dissolving sodium aluminate (anhydrous, Riedel de Haen) in a hot solution of sodium hydroxide (J.T. Baker) and deionized water. After cooling to room temperature, tetraethylammonium hydroxide (Acros Organics, 20%) was added into the mixture in appropriate proportion and stirred for 15 min at room temperature. The silica containing precursor was prepared by mixing Ludox HS-40 (Sigma–Aldrich) with deionized water. Then alumina and silica sources were mixed and placed in a 140 °C oven for 14 days.

Silicalite was synthesized with the molar formula of $TPAOH{:}5TEOS{:}500H_2O$. Tetraethylorthosilicate (TEOS, Acros Organics, 95%) was used as the silica source. Tetrapropylammoniumhydroxide (TPAOH, Acros Organics, 25%) was used as a template. The mixture of TEOS and TPAOH was continuously stirred for 6 h at room temperature. The resulting gel was placed in an oven for 18 h at 125 °C. The solid particles obtained from the synthesis of silicalite and zeolite Beta were centrifuged at 13,000 rpm, washed with deionized water, and dried at 80 °C. Silicalite particles with an average size of ~0.5 μm and zeolite Beta particles with an average size of ~0.9 μm were synthesized according to the explained procedures.

Urease (activity index of 22 U/mg, Sigma), bovine serum albumin (BSA, fraction V) and glycerol (minimum 99%, Sigma), 50% aqueous solution of glutaraldehyde (GA) was purchased from Fisher Scientific. Phosphate buffer solution (KH_2PO_4–Na_2HPO_4) was used as a working buffer. The compounds for buffer preparation as well as other inorganic compounds used were of analytical grade.

2.2. Construction of enzyme electrodes and zeolite modified electrodes

Three different types of electrodes were constructed in the current work. General scheme of all electrodes are given in Fig. 1.

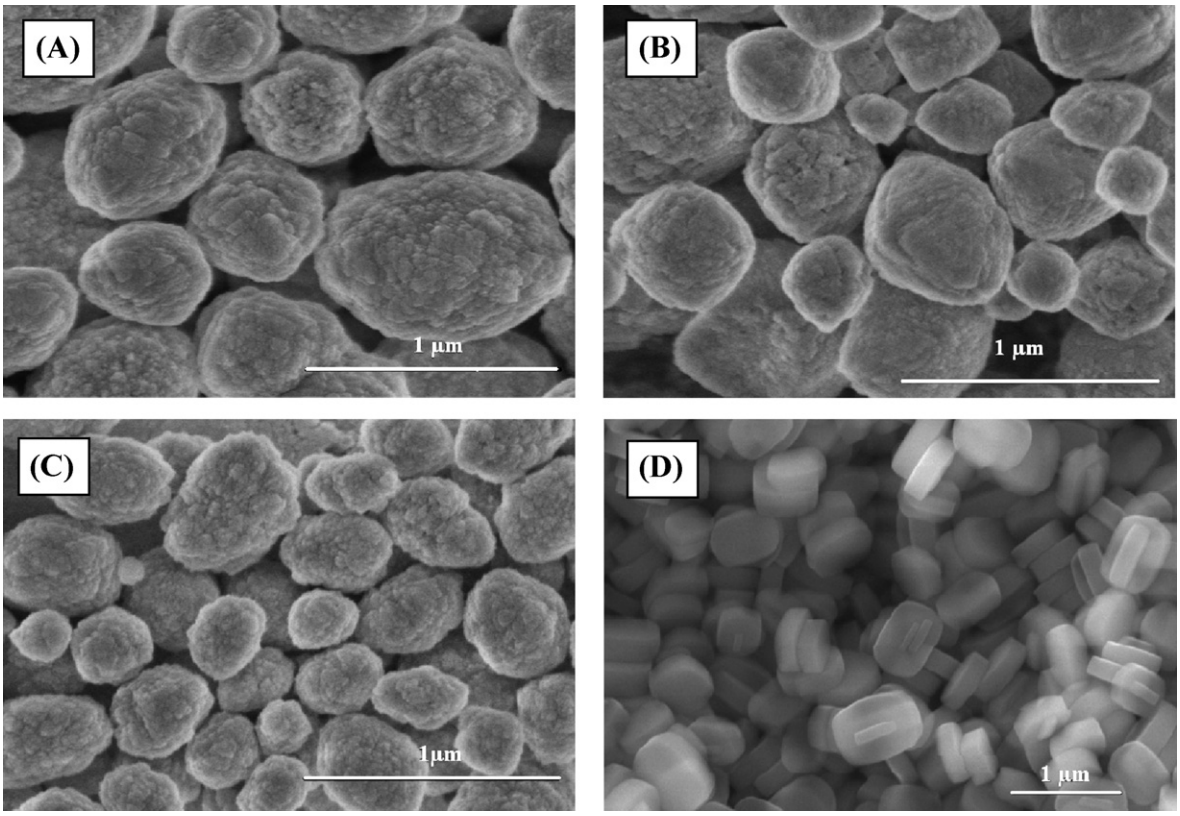

Fig. 2. SEM images of zeolite Beta 40 (A), Beta 50 (B), Beta 60 (C) and silicalite-1 (D).

The conductometric transducers were produced in Lashkarev Institute of Semiconductor Physics of National Academy of Sciences of Ukraine. They consisted of two identical pairs of gold interdigitated electrodes made by gold vacuum evaporation onto pyroceramic substrate (5 mm × 40 mm). The surface of sensitive area of each electrode pair was about 1.0 mm × 1.5 mm. The width of each of interdigital space and digit was 20 μm.

The first type of electrode, which is called as Standard Membrane Transducer (SMT) contains no zeolite and was constructed typically as shown in Fig. 1A. The solution used to prepare the working membrane contains a mixture of 5% urease, 5% BSA, 10% glycerol in phosphate buffer solution (PBS), while the solution used to prepare the reference membrane contains a mixture of 10% BSA, and 10% glycerol. Then transducers were kept under glutaraldehyde vapor for 35 min.

The second type of electrode, which is called as Zeolite Membrane Transducer (ZMT) contains zeolite particles that were simply added to the immobilization mixture as shown in Fig. 1B. Accordingly, the solution used to prepare the working membrane contains a mixture of 5% zeolite, 5% urease, %5 BSA, 10% glycerol in PBS, while a mixture of 5% zeolite and 10% BSA in PBS without any enzyme was used for the reference membrane. Then transducers were kept under glutaraldehyde vapor for 35 min.

The third and the final type of electrode was called as Zeolite Coated Transducer (ZCT) and was developed from zeolite thin films prepared on gold electrodes of conductometric biosensors as shown in Fig. 1C. The transducer surfaces of both working and reference electrodes were modified by dip coating the surfaces with 5% zeolite suspension. Then 0.1 μl, 5% urease solution in phosphate buffer solution was dropped on the working electrode side and 0.1 μl, 5% BSA in PBS was dropped on the reference electrode side of the electrode. The electrodes were not exposed to glutaraldehyde vapor for this modification route.

2.3. Electrochemical measurements

The electrochemical measurements were performed with an electrochemical device (Stanford Research Systems Model SR830 Lock-In Amplifier) connected to a PC through the serial port. All of the experiments were carried out using a 5 mL beaker filled with 5 mM PBS of pH 7.2. The substrate (Urea) added is 1 mM in each reading. Every experiment was repeated for 5 times.

2.4. Characterization

To determine the physical characteristics of each material, nitrogen adsorption–desorption isotherms were measured at 77 K on a Quantachrome Corporation, Autosorb-6. The zeolites were degassed at 300 °C under high vacuum for 4 h prior to the nitrogen adsorption measurements. The average pore size was taken as the peak of the pore size distributions as calculated from the adsorption branch using the Barrett–Joyner–Halenda (BJH) method. The total pore volume was determined as the volume of liquid nitrogen adsorbed at P/P_0 of 0.995.

SEM measurements were done using FEI Quanta 400F Field Emission scanning electron microscope, operated at 30 kV. The energy dispersive X-ray spectroscopy (EDX) analysis of all samples for Si/Al ratio determination was carried out utilizing an EDAX X-ray analyzer equipped with a Lithium doped silicon detector attached to the FEI Quanta FE-SEM. The characterization results are given in Table 1. SEM results of the synthesized zeolites depicted in Fig. 2 show that the prepared zeolites are about ~0.8 μm in diameter with a narrow/large particle size distribution for zeolite Beta and ~0.5 μm for silicalite samples. Fig. 3 displays a typical Zeolite Coated Transducer (ZCT) upon modifying the electrode surface with silicalite and zeolite Beta.

Table 1
Surface properties of examined zeolites.

Materials	Si/Al ratio	S_{BET} (m^2/g)	Particle size (nm)	Pore size (Å)	Pore volume (cc/g)
BEA40	12.9	349	0.79	5.559	8.680
BEA50	21.2	462.2	0.83	5.260	9.593
BEA60	24.5	376.1	0.91	4.906	7.109
Silicalite	No Al.	281.7	0.50	5.221	2.014

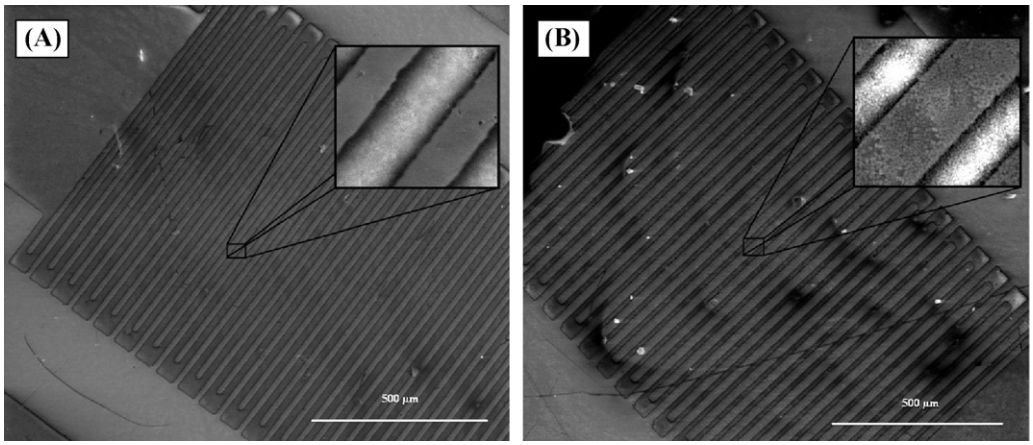

Fig. 3. SEM images of the Zeolite Coated Transducers (ZCT) with silicalite (A) and BEA60 (B).

3. Results and discussion

3.1. Response characteristics of the biosensor to urea on silicalite modified thin-film conductometric electrodes

The response curves of the conductometric biosensors as a function of time upon addition of urea is also shown in Fig. 4. As shown in Fig. 4, after the biosensor reached a stable response value in blank phosphate buffer solution, injection of urea stock solution caused significantly faster sensor response in ZCT electrodes. This might be due to GA layer on top of the transducer in SMTs, which may be considered as a diffusion barrier. Full response was reached for SMT in ca. 80 s, while it only took about 8 s to reach the full performance for the ZCT biosensors. These results were significantly shorter than the ones obtained by Lee et al. using silica sol–gel matrix in which they obtained steady state values in 16.5 min [4]. They claimed that the long response times were due to the relatively thick sol–gel films of 25 ± 2 μm. In the current study, the thicknesses of the films were measured as 5 ± 1 μm. Thus, the applied methodology in this work

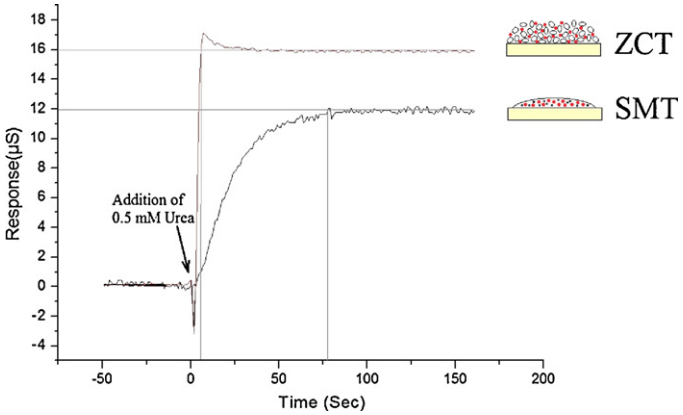

Fig. 4. Response curves of conductometric biosensor based on urease ZCT and SMT.

to modify electrode surfaces also gives the advantage to coat the surfaces in a more controlled manner with respect to the traditional sol–gel methodologies. Even only a single crystal thickness could be attained on various electrode surfaces if desired [21,22]. It can be hypothesized that the reason for the observed significant decrease in the time spent to reach equilibrium response values after the injection of urea is again due to the enhanced effective spaces of the surface of modified electrodes for enzyme immobilization. This property, in addition to the hypothesized biocompatibility of zeolitic materials can be important for applications, which require fast responding sensors.

Zeolite coated conductometric thin-film electrodes were investigated with urease for urea determination. It was seen that, preparation of Zeolite Coated Electrodes by silicalite which means modifying the electrode surfaces before getting any biosensor measurements lead to an increased response in comparison with the Standard Membrane Transducers (SMTs). This increased response was significantly higher for ZCT (27.5 μS) with respect to SMT (16.8 μS) type electrodes for 8 mM urea injection. Dynamic ranges for urea determination were nearly same for both types of conductometric biosensors.

Since selectivity is very important characteristic of biosensor, it was necessary to check this characteristic of biosensor developed. It was shown that conductometric urease biosensor has demonstrated a high selectivity to urea alone with no response to uric acid, creatine, glucose and creatinine as substrates alone.

Although, this had been a first time investigation of such an approach for modified electrodes in urea measurements using conductometric biosensors, the effect of adding zeolites into the enzyme containing membranes was studied using different zeolites and electrochemical biosensors. The effect of zeolite addition on enzymatic activity using NaY as the zeolitic material and cutinase as the enzyme was deeply investigated using fluorescence emission spectra by Vidinha et al. [23]. Their results indicated that placing the zeolite in close proximity to the enzyme improved the accessibility of the enzyme to the substrate and lead to higher enzymatic activity. Zhou et al. [7] also made a similar discussion

constructing a layer-by-layer ITO electrode surface using zeolite Beta for the adsorption of enzymes and measuring their amperometric responses. They proposed that zeolite addition enhanced the effective spaces of the surface of modified electrodes for enzyme immobilization. In the current study, the conductometric electrode surfaces were modified by silicalite type zeolites for the first time for urea determination and similar enhancements were observed for ZCT type electrodes.

To have a full understanding of the biosensor related properties of the ZCT type conductometric electrodes, operational and storage stabilities were also investigated.

3.2. Operational and storage stability of ZCT with silicalite

The practicability of biosensors is often limited by its operational and storage stability. For the ZCT electrodes, the operational and storage stabilities were tested over a 400 min and 2 month periods, respectively by monitoring the responses to the injection of 1 mM of urea. In the operational stability experiments, it was seen that Zeolite Coated Transducers retain their initial activities after 6 h. Also storage stability of the sensors was tested and it was seen that the prepared Zeolite Coated Transducers lose 50% of their activities in a week, but after that, they retains their activity for a month. Lee et al. [3,4] reported that conductometric sol–gel immobilized urease biosensor used with commercial urea stored 50% of its original activity after 3 weeks in serum. Although the storage stability results were obtained in model solution in the current study, the obtained results are still comparable with the ones obtained by Lee et al.

The adsorption of proteins on inorganic substrates can be seen as a simple phenomenon; however there are many different parameters that can be effective in this matter [24]. The interaction of enzymes with zeolites can actually be complicated due to different factors, such as hydrophilic and hydrophobic, electrostatic, and/or structural interactions. Tavolaro et al. [24] showed that protein adsorption on zeolites can be influenced by the Brönsted acidity of the zeolite. It is well known that enhanced catalytic activities can be gained through a controlled variation in the number and strength of framework Al–OH–Si groups (i.e., Si/Al ratio) that are known to be the Brönsted acid sites in zeolites. There are various studies in the literature trying to investigate the interaction between the zeolites and proteins on a systematic basis by changing the acidic properties of zeolites, however these investigations are usually made by changing the zeolite type, and thus the zeolite structure [24]. On the other hand, once the structure is changed hoping to alter the acidity of the zeolite for such an investigation, many different parameters are also changing, like the overall nature and density of the defect sites, the external surface area, surface roughness and morphology, pore sizes, etc.

Accordingly, in the current study, it was aimed to make a more systematic investigation on the effect of Brönsted acidity in terms of whether it really has an influence or not on the obtained activities on the ZCT conductometric biosensors by using the same type of zeolite, which is zeolite Beta. Zeolite Beta, a wide-pore zeolite, can be ideally used to study a wide range of Si/Al ratio without the necessity to change the zeolite type to obtain varying Brönsted acidities in the same structure [25]. Thus, comparison of conductometric urea biosensor responses obtained using ZCT type electrodes was performed using zeolite Beta with three different Si/Al ratio of 40, 50, and 60 in the current study for the first time.

3.3. Effect of Si/Al ratio on conductometric urea biosensors

In this study, a new method as shown in Fig. 1C and denoted as "ZCTs" was tested and studied to modify conductometric transducer surfaces using zeolite Beta to investigate the effect of changing Si/Al ratio. The results obtained were compared by the "Standard Membrane Transducers" (SMTs) which contains no zeolite and Zeolite Membrane Transducers (ZMTs) where the enzymatic solution contains zeolite Beta nanoparticles as an alternative technique. In general, it was aimed to investigate whether the prepared zeolite film on the transducers were going to maintain surface characteristics of zeolite Beta nanoparticles by observing whether different and especially consistent responses were going to be obtained as a function of changing Si/Al ratio. In this method, surfaces of the transducers were directly coated with zeolite Beta nanoparticles with Si/Al ratio of 40, 50, and 60. Then 0.1 μl, 5% urease solution in phosphate buffer solution was dropped by a micropipette onto the working electrode side and 0.1 μl, 5% BSA in PBS was dropped onto the reference electrode side of the electrode. By doing so, gluteraldehyde was avoided, which is known to inactivate the enzymatic activity of membrane [26].

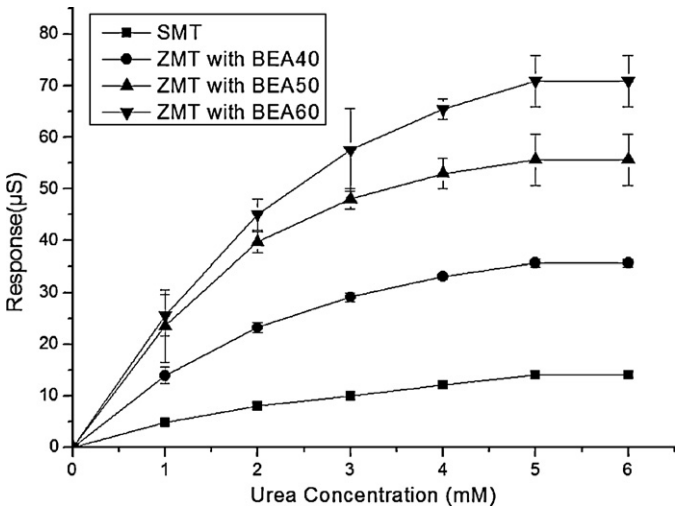

Fig. 5. Comparison of responses obtained using ZMT's prepared using BEA40, BEA50 and BEA60 and SMT type conductometric biosensors.

For this purpose, firstly "zeolite modified transducers" (ZMTs) were tested against the "Standard Membrane Transducers" (SMTs) for zeolite Beta with varying Si/Al ratio (BEA40, BEA50, and BEA60) mixed enzymatic membranes on the conductometric biosensors. The comparison of calibration curves obtained among ZMT's with BEA40, BEA50 and BEA60 and SMT's are shown in Fig. 5.

The Si/Al ratio of zeolites is used to denote the hydrophobicity of zeolites, with higher ratios indicating a higher degree of hydrophobicity and lower ion-exchange capacity. The morphology of the zeolite structure varies [20].

In this process, interactions between the support and the guest molecules are of non-covalent nature, such as hydrogen bonding, electrostatic, van der Waals and hydrophobic or hydrophilic interactions, thus relatively weak. The immobilization techniques, however, could affect their catalytic activity.

Fig. 5 shows the conductometric enzymatic responses of all ZMT and SMT electrodes obtained after the addition of urea solution into 5 mM phosphate buffer solution. According to Fig. 5, all ZMT's showed higher responses with respect to the traditional SMT's. It is known that for large zeolite particles, the outer surface of zeolite crystals possesses about 5–10% of the total zeolite surface. Even for such cases, there will still be a relatively large surface interaction between zeolite and biological species due to the highly dispersed zeolites in the membrane [27]. Accordingly, it can be seen that there had been an increased interaction between the zeolite nanoparticles and the enzyme leading to an increased response obtained using all ZMT's.

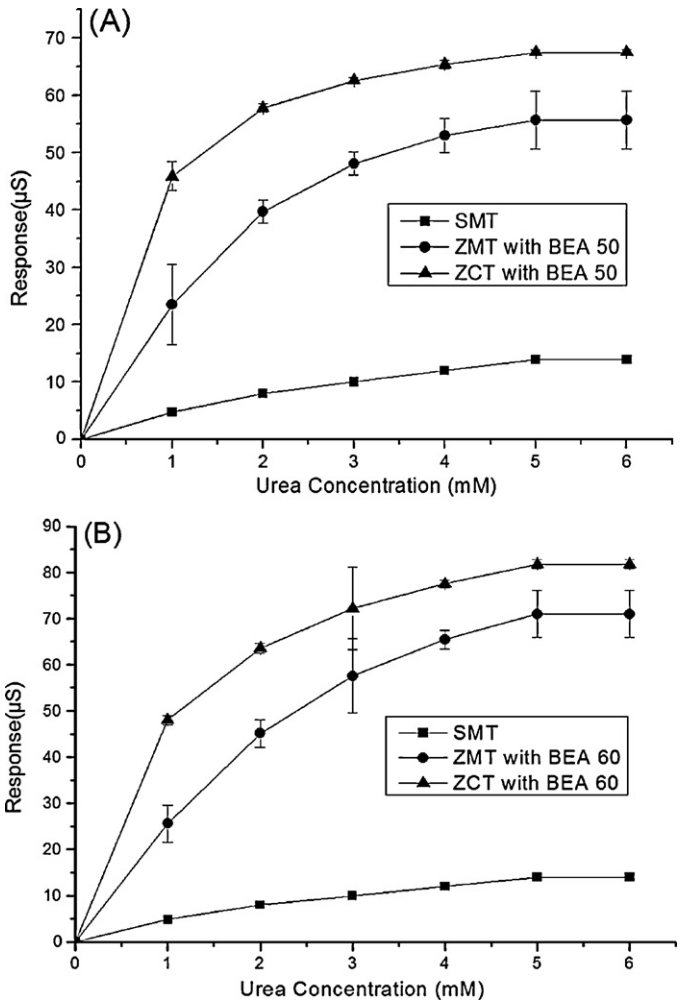

Fig. 6. Comparison of the calibration curves between SMT, ZMT and ZCTs using zeolite BEA50 (A) and BEA60 (B).

Furthermore, it can be seen that the conductometric responses increased with increasing Si/Al ratio for ZMT type electrodes. As shown in Fig. 5, the highest response was obtained from BEA60 and the lowest from BEA40, with the medium response obtained from BEA50. This correlation also indicates that there had really been some sort of interaction between the zeolite nanoparticle surface and the enzyme for each particular case. This behavior can be due to an increased hydrophobicity and/or the increasing acidic strength with the increasing Si/Al ratio within the zeolite crystals. Mintova et al. [28] clearly discussed the results showing that high Al containing zeolite HZSM-5 possessed more hydrophilic active sites, while the zeolites with low Al content showed the opposite characteristics. Accordingly, the changing Brönsted acidity and the hydrophilicity of zeolites are interrelated and cannot be discussed as separate factors affecting the protein adsorption on zeolites. The studies showed that acid strength increase with the decrease of the aluminum atoms in the zeolite [26,29]. Furthermore, in the literature it was seen that number and the mobility of the zeolitic cations play an essential role in charge transfer across the membrane phase and do significantly influence the responses [30]. Accordingly, the results obtained and shown in Fig. 5 clearly demonstrate the changing biosensor response as a function of acidity and the hydrophilicity of zeolite Beta for conductometric urea biosensor for the first time.

The effect of directly modifying the electrode surface with different types of zeolite Beta type zeolites was also investigated to study whether a similar response correlation was going to be obtained as a function of Si/Al ratio and if the ZCT and ZMT responses were going to be different than each other for each type of zeolite. Accordingly, the conductometric responses obtained using BEA50 and BEA60 is shown for ZMT and ZCT type electrodes in comparison with the responses obtained on the SMT and the results are shown in Fig. 6.

As shown in Fig. 6, Zeolite Coated Transducers (ZCTs) gave higher responses than those of SMTs and ZMTs. Relative standard deviation of the output signal found as 5% for SMTs, 6.7% for ZMTs and 3.6% for ZCTs in this study. The membranes in SMT and ZMT technique have very low reproducibility due to cast a very thin film on transducer surface and it is almost impossible to cast the same film in every trial. When compared with SMTs and ZMTs, ZCTs have higher reproducibility due to the controlled thickness of zeolite thin film by dip coating, and the known amount of enzyme adsorbed to this film. Moreover, glutaraldehyde which can be considered as a diffusion barrier and a poisonous chemical for enzymes is not used in ZCTs.

4. Conclusion

In this work, a new aproach to electrochemical biosensors introduced as Zeolite Coated Transducers (ZCTs). Silicalite and zeolite Beta with Si/Al ratios 40, 50 and 60 are used to produce Zeolite Membrane Transducers (ZMTs) and Zeolite Coated Transducers (ZCTs). Effect of Si/Al ratio is studied with different surface modification techniques such as Zeolite Membrane Transducers (ZMTs), Zeolite Coated Transducers (ZCTs) and compared with Standard Membrane Transducers (SMTs). Increasing Si/Al ratio in zeolite Beta gave higher responses in both ZMTs and ZCTs, due to increasing hydrophobicity and acid strength may influence the adsoption on zeolites. Also Zeolite Coated Transducers gave higher responses than both SMTs and ZMTs. The reason behind this can be explanied by the lack of the glutarladehyde which can be considered as a diffusion barrier, and better interaction between zeolite and enzyme.

This new aproach can be applicable to all kinds of electrochemical biosensors since it's easy to produce this transducers, it has good storage and working stability and it enhances signals.

Acknowledgments

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