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Impedimetric Urea Biosensor Based on Modified Gold Electrode with Urease Immobilized on Glutathione Layer

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Abstract: In this work, a glutathione (GSH) modified gold microelectrode was used for the covalent immobilization of urease biomolecules via the glutaraldehyde-coupling agent. The self-assembled monolayers (SAMs) onto the gold surface was investigated by using the electrochemical impedance spectroscopy measurements (EIS). Before urease grafting, a significant interaction was noticed between urea and the glutathione layer by forming hydrogen bonds. The H-NMR analysis was carried out to highlight the possibility of having a covalent link between urea and the GSH deposited layer. In addition, contact angle measurements were carried out to determine the hydrophobic/hydrophilic feature of the modified gold surface electrode. After urease immobilization a stable and high sensitive impedimetric urea biosensors was obtained with a sensitivity of 8.73 10^{-8} $\Omega^{-1}mM^{-1}$ for the low concentrations range and a sensitivity of 7.03 10^{-9} $\Omega^{-1}mM^{-1}$ for the high concentrations range.

Keywords: Glutathione SAMs, Urea-GSH interaction, Urease immobilization, Impedance spectroscopy, Urea biosensor.

1. Introduction

Some chemical and biochemical target molecules present a major role in the human life continuity and have considerable interest in clinical and agricultural chemistry. When the concentration of these substances becomes higher than the normal level a permanent supervision should be established. In fact, both robust and miniaturized instruments named enzymatic biosensors are astonishing the researchers worldwide for the reason that they are required in the analysis of several biological substrates such as glucose [1], cholesterol [2], penicillin [3], acetylcholine [4], creatinine [5], urea [6], adrenaline [7], dopamine [8]. As an example, urea is of a biological interest and is widely found in nature such as soil [9], milk [10], alcoholic beverages [11] and urine [12]. Urea is defined as an important label for evaluating the uremic toxin levels (The normal level of urea in serum is from 15 to 40 mg/dL). That's why its analysis in the human body, in food chemistry and environment monitoring is getting bigger importance and urease biosensor is the best innovation to achieve this aim. Up to now many urea biosensors were developed including potentiometric [13], amperometric [14], impedimetric [15],

couloumetric [16], piezoelectric [17], optical [18], conductimetric [19] and thermal [20] methods. Despite the multitude of these created biosensors, it still in the area of scientific laboratories but with a constant hope to be commercialized. Actually, the major problem which handicaps the electrochemical success of these biodevices in urea monitoring depends essentially on the urease biomolecules and the transducer biocompatibility. This leads to the best integrity between the basic components of the biosensor and preserves the enzymatic activity. No one could deny that urease is a highly specific enzyme which catalyses the hydrolysis of urea to ammonium and hydrogenocarbonate resulting to an increase in pH of the reaction environment as follows [21].

$$CO(NH_2)_2 + 3\,H_2O \xrightarrow{Urease} 2NH_4^+ + HCO_3^- + OH^- \quad (1)$$

This enzymatic reaction takes place in a small zone located in the urease biomolecule called "pocket" or active site [22]. The latter presents both physical and chemical complementarities serving for the specific recognition and the degradation of the urea respectively. To maintain this specific characteristic of urease after its immobilization, many strategies have been investigated based on different immobilised procedures which contain basically physical adsorption and covalent attachment methods. This can be established by making direct covalent link between urease and the material transducer support. Otherwise a bifunctional reactive intermediate like glutaraldehyde [23] and carbodiimide [24] is extensively used for enzyme attachment and consequently prevent it from the chemical and mechanical denaturation. As an example, glutaraldehyde coupling-agent is so much used in cooperation with aminothiol [25] or aminosilane [26] derivatives for the enzyme attachment. This is due to the specific and spontaneous reaction between amino (-NH_2) and aldehyd (-COH) groups [27]. Thiol (-SH) terminated molecules as well bond easily onto metal surfaces such as platinum and gold [28, 29]. The same for silanol groups which bond onto silicon dioxide (SiO_2) to form nano-structured and thin organic layer called self-assembled monolayer (SAM). This assembly is characterized by high stability and low molecular defect density [30]. For example, self-assembled monolayers of thiolated molecules are used in the development of different systems which are important for the enzyme immobilization. In this work, impedimetric urea biosensor was investigated being based on the self-assembled monolayer method. Urease biomolecules were immobilized on gold microelectrode modified with glutathione reduced form using the glutaraldehyde reticulation process.

The electrochemical impedance spectroscopy, H-RMN and contact angle measurements were carried out in order to characterize the analytical functioning and to control the building layer by layer of the bioarchitecture

2. Experimental Section

2.1. Reagents

Urease from jack beans (57 U/mg), glutaraldehyde and urea were obtained from Sigma. Glutathione reduced form (GSH) with purity of 98 % was purchased from Aldrich and was used without further purification. Other reagents and chemicals were of analytical grade. A gold electrode made of a 300 nm thick gold film on an insulated silicon substrate purchased from LAAS-CNRS, Toulouse-France was used as working electrode (0.078 cm^2 geometrical area) and was thoroughly cleaned by chemical treatment before the surface modification.

2.2. Urease Immobilization and Assembling of the Biosensor

To enhance the gold surface reactivity towards glutathione S-H groups the assembling of the biosensor layer by layer begins by a cleaning step.

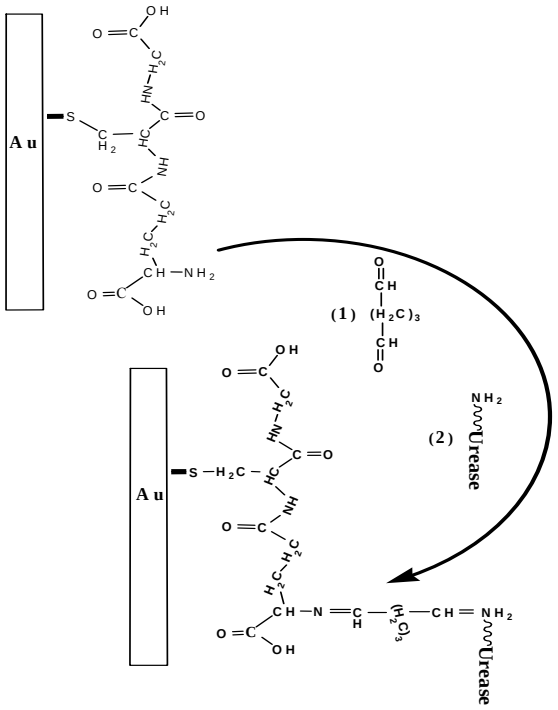


Fig. 1. Schematic representation of the immobilization of urease on gold electrode using a SAM of Glutathion via Glutaraldehyde as coupling agent.

In fact the gold surface being treated using acetone ultrasonic bath for 10 min, piranha mixture for 1 min and dried under nitrogen flow at room

temperature. After the cleaning step the gold microelectrode was modified by dip-treatment in ethanol solution containing 10 mM of glutathione for 3 h.

After the modification step, the microelectrode was thoroughly rinsed with pure ethanol and then with water to remove non covalent linked glutathione molecules. Then, the modified electrode was exposed to saturated vapors of glutaraldehyde for 1h before to be immersed in the urease solution (2 mg/mL) for 3h at 4 °C. In fact, glutaraldehyde is a bifunctional agent containing –COH groups react immediately with available amino groups leading to cross linking of glutathione and urease biomolecules through Schiff bases (–N=CH–). Fig. 1 shows the Schematic representation of the immobilization of urease on the gold electrode.

2.3. Apparatus

2.3.1. Contact-angle Surface and ^{1}H-NMR Spectroscopy Characterizations

Contact-angle measurements consisted of the sessile drop method with an apparatus provided by GBX scientific instrument (Romans, France). Image of the drop deposit on the modified gold electrode surface (1 cm^2) was recorded by a video camera and an image-analysis system calculates the contact-angle (θ) from the shape of the drop. ^{1}H-NMR spectra were recorded on a Bruker Avance DPX 250 (250 MHz for the 1H-NMR) as d_6-Me_2SO solutions.

2.3.2. Electrochemical Impedance Spectroscopy Measurements

The electrochemical impedance spectroscopy (EIS) was used to investigate the biosensor response to urea addition at ambient temperature. The Urease/Glutathione coated gold microelectrode was connected to an Autolab PG301 electrochemical analyzer system (Eco Chemie, Netherlands) with a FRA2 module. The EIS electrochemical measurements were performed in a conventional electrochemical cell containing a three-electrode system, ensuring stable positioning of the electrodes and an agitation of the solution.

The modified gold microelectrode was the working electrode, a platinum wire the auxiliary electrode and a saturated Ag/AgCl/KCl electrode served as reference electrode. Impedance measurements were performed in the frequency range from 0.1 to 100,000 Hz with signal amplitude of 10 mV.

The urea biosensor response were recorded in 20 mM phosphate buffer solution (pH=7.0) containing 0.1M of NaCl. All measurements were performed in a dark Faraday cage.

3. Results and Discussion

3.1. Investigation of the Hydrogen Interaction Between the Grafted Glutathione Layer and Urea Molecules

Electrochemical impedance spectroscopy (EIS) is becoming a very useful method for modified solid-liquid interface interpretation [31]. This technique provides much information on the impedance change of the electrode surface during the fabrication process. In this report glutathione modified gold surface was characterized by EIS measurements towards urea addition.

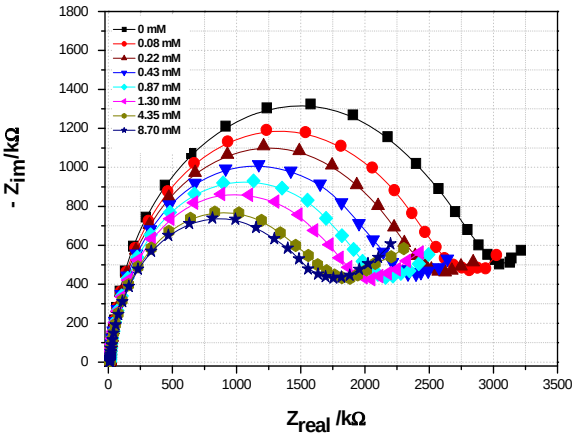


Fig. 2. Electrochemical impedance spectra of the Glutathione modified gold electrode in the presence of different urea concentrations. 20 mM phosphate buffer containing 0.1 M of NaCl and pH=7.4, applied potential -0.3 V.

Fig. 2 shows the impedance spectra of the modified gold microelectrode for different urea concentrations. The impedance signal has a semi circle geometrical form for a polarization potential of -0.3 V. As can be seen, the diameter of the semi circle decreases when the urea concentration increases in comparison with the bare gold microelectrode that shows a negligible response. This result can be attributed to the interaction between urea and glutathione through hydrogen bonds. After the glutaraldehyde reticulation process the modified gold microelectrode remains sensible for urea addition in spite of the desactivation of the glutathione amine groups (Fig. 3).

This residual sensitivity to urea addition can be attributed to the interaction between urea and the glutathione nitrogen atoms no involved in the reticulation process. In a previous work an astonishing interaction between urea and glutathione was established. It was demonstrated that this high interaction played any significant part in the activity of human synthetic saliva [32]. In Fig. 4 we proposed a conceptual scheme of the Glutathione-Urea interaction via hydrogen bonds.

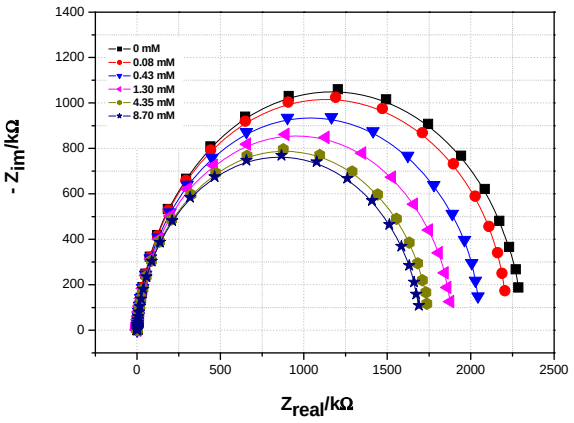


Fig. 3. Electrochemical impedance spectra of the Glutaraldehyde-Glutathione modified gold electrode in presence of different urea concentrations. 20 mM phosphate buffer containing 0.1 M of NaCl and pH=7.4, applied potential -0.3 V.

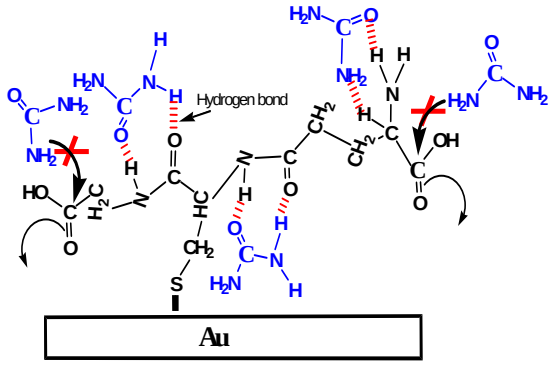
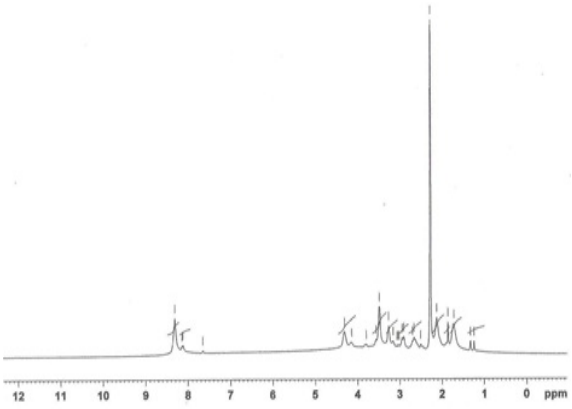


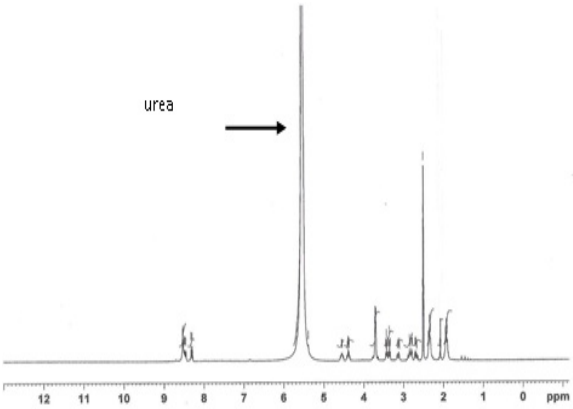
Fig. 4. A conceptual scheme of the Glutathione-Urea interaction via hydrogen bonds.

3.2. Investigation of Chemical Interaction Between Glutathione and Urea by ^{1}H-NMR Study

Our work is devoted to immobilize urease on gold surface modified with glutathione molecules for urea monitoring. It is known that Glutathione is a biological compound containing thiol, amine and carboxylic free groups. As it is shown in Fig. 1 the thiol groups react spontaneously with the Au surface. In addition, glutarldehyde as a bifunctional agent react immediately with the free amine groups of glutathione and urease biomolecules. The unused glutathione carboxylic groups remain alert and it can react with nucleophilic agent like urea in our work. For this reason nuclear magnetic resonance technique was carried out to elucidate the possibility of chemical interaction between urea and glutathione. In Fig. 5 we report the ^{1}H nuclear magnetic resonance (^{1}H-NMR) (JEOL; EX-270) spectra of glutathione with (spectrum b) and without (spectrum a) urea. As a result, we could observe no change in glutathione spectrum after urea addition confirms the non covalent link that can be established between urea and glutathione. We conclude that urea interacts with glutathione through hydrogen bonds and the covalent interaction was probably absent.



(a)



(b)

Fig. 5. ^{1}H-NMR spectra of Glutathione: (a) before, and (b) after urea addition.

3.3. Multilayer Assembly and Contact Angle Characterization

The thermodynamic features like hydrophobicity, hydrophilicity or the free surface energy and its components can be quantified by contact angle measurements [33]. In our work experimental measurements were carried out using the sessile drop method. The change of the surface polarity before and after such modification step as well as the quality of such formed monolayer can be estimated from the wetting measurements; the shape of the liquid drop is affected by the free energy of this modified surface. The obtained contact angle value using water as test liquid probe was found to vary from 82° for the untreated blank gold surface, 85° when the gold surface is treated with acetone (10 min), and 60° after piranha treatment (1 min). It is important to note here that the increase of the contact time between the gold surface and the piranha mixture leads to a low

contact angle value. In our case one minute is sufficient because gold layer is very thin and risky to be distorted by the piranha treatment. This tendency in contact angle change was in good agreement with the impedance results obtained from the same treated gold surface (results not shown here). A decrease in contact angle was observed (30°) when the gold surface was grafted with glutathione which indicates the formation of the SAM layer. This hydrophilic behaviour observed for the deposit glutathione layer can be attributed to the presence of the amine and the carboxylic polar groups which lead to a high wettability surface. This behaviour appears contrary to other more hydrophobic thiol layers grafted on the gold surface such as Ethanethiol (67°), Propanethiol (69°), Pentanethiol (86°), Dodecanethiol (105°) and 6-Mercapto-1-hexanol (54°) [34]. Fig. 6 shows the contact angle change for the gold surface before and after such modification step. A high change in the contact angle value near 74° was observed after the glutaraldehyde reticulation.

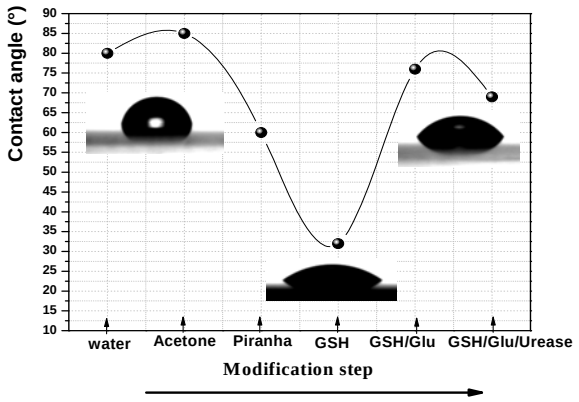


Fig. 6. Static contact angle variation during the building of the SAM system on the gold electrode surface.

After urease immobilization a contact angle value near 69° was obtained confirms its binding onto the glutathione modified gold surface through the glutaraldehyde cross-linked agent.

3.4. Sensitivity of the Urease/Glutaraldehyde/ Glutathione/Au Enzymatic Microelectrode.

The development of a biosensor for biological analysis not only requires that the thiolated layer has good and high affinity for enzyme immobilization, but also that this layer shows good biocompatibility. Glutathione has this latter characteristic and is often used for biological or medical applications. In our work urease was immobilized by self-assembled monolayer strategy and was immediately applied for urea catalysis assay. The sensitivity of the constructed urea biosensor was evaluated by using electrochemical impedance spectroscopy measurements. Different volumes of urea were corresponding to different concentrations in the cell and were added at room temperature. In Fig. 7 we report the impedance spectra of the modified gold microelectrode for urea addition.

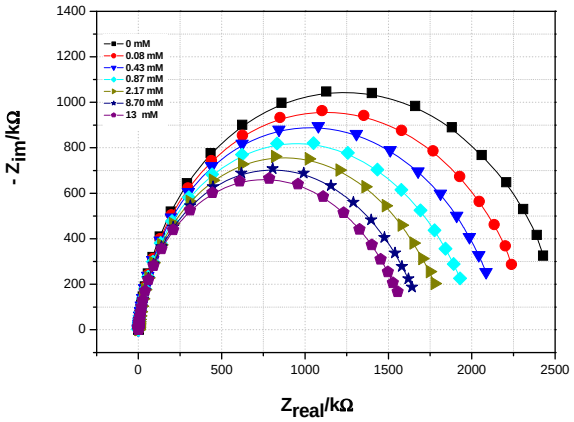


Fig. 7. Electrochemical impedance spectra of the Urease-Glutaraldehyde-Glutathione modified gold electrode in presence of different urea concentrations. 20 mM phosphate buffer containing 0.1 M of NaCl and pH=7.4, applied potential -0.3 V.

Indeed, as the products of the urea hydrolysis are alkaline, an increase of urea concentration in the analyzed solution causes an increase of pH inside the enzyme layer of the biosensor. Then this variation of pH causes a variation of the electrode impedance which can be detected. We observe that the impedance measurement results of urea in range of 0-13 mM. The semicircle diameter in the Nyquist plot seems to decrease with the urea concentration. Fig. 8 shows the biomembrane resistance differences versus the added urea concentration. As we can see, the sensor is sensitive to urea addition.

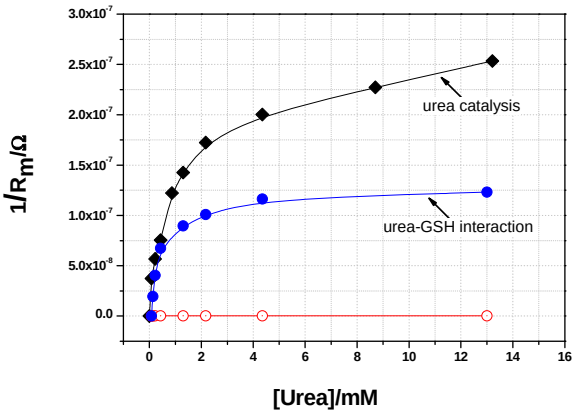


Fig. 8. Calibration plots of the membrane resistance variation as a function of the urea addition. (⊙) bare, (●) Glutathione/Au and (◆) urease/ glutaraldehyde/Glutathione/Au electrodes. 20 mM phosphate buffer containing 0.1 M of NaCl and pH=7.4.

Under optimal conditions, i.e. buffer capacity corresponding to 20 mM phosphate buffer containing 0.1 M of NaCl and pH=7.4, the urea biosensor showed an impedimetric linear response with a sensitivity of 8.73 10^{-8} $\Omega^{-1}mM^{-1}$ for the low concentrations range and a sensitivity of 7.03 10^{-9} $\Omega^{-1}mM^{-1}$ for the high concentrations range. The obtained urea biosensor response can be attributed to the specific and non specific interaction between urea and immobilized urease on glutathione layer. In one hand the non specific interaction can be explained by the urea-glutathione interaction through hydrogen bonds. In other hand the specific interaction was due to the catalytic process based on the substrate-enzyme interaction leading to the urea degradation.

4. Conclusions

In this work an impedimetric urea biosensor was developed based on the self assembly process on gold microelectrode. The constructed SAM system was successfully tested for the impedimetric sensing of the urea in solution. Before urease immobilization we have highlighted the possibility of interaction between urea and glutathione via hydrogen bonds. Using the contact angle measurements the hydrophobicity and the hydrophilicity of the gold surface was attempted before and after such grafted layer. Under optimized conditions the developed urease-Glutathione-Au bioelectrode was tested to urea addition under electrochemical impedance spectroscopy measurements. As a result, a high sensitivity and good stability of the bioelectrode were observed during the urea addition with a sensitivity of 8.73 10^{-8} $\Omega^{-1}mM^{-1}$ for the low concentrations range and a sensitivity of 7.03 10^{-9} $\Omega^{-1}mM^{-1}$ for the high concentrations range.

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References

[1]. T. Kong, Y. Chen, Y. Ye, K. Zhang, Z. Wang, X. Wang, An amperometric glucose biosensor based on the immobilization of glucose oxidase on the ZnO nanotubes, *Sensors and Actuators B*, Vol. 138, 2009, pp. 344-350.

[2]. S. Brahim, D. Narinesingh, A. Guiseppi-Elie, Amperometric determination of cholesterol in serum using a cholesterol oxidase biosensor with a polypyrrole / hydrogel membrane, *Analytica Chimica Acta,* Vol. 448, 2001, pp. 27-36.

[3]. M. Stredánský, A. Pizzariello, S. Stredánská, S. Miertuš, pH-sensing biosensors for urea, penicillin and oxalacetate, *Analytica Chimica Acta,* Vol. 415, 2000, pp. 151-157.

[4]. T. Shimomura, T. Itoh, T. Sumiya, F. Mizukami, M. Ono, Amperometric biosensor based on enzymes immobilized in hybrid mesoporous membranes for the determination of acetylcholine, *Enzyme and Microbial Technology,* Vol. 45, 2009, pp. 443-448.

[5]. S. Yadav, A. Kumar, C. S. Pundir, Amperometric creatinine biosensor based on covalently coimmobilized enzymes onto carboxylated multiwalled carbon nanotubes/polyaniline composite film, *Analytical Biochemistry,* Vol. 419, 2011, pp. 277-283.

[6]. H. Barhoumi, A. Maaref, M. Rammah, C. Martelet, N. Jaffrezic-Renault, C. Mousty, S. Cosnier, E. Perezet I. Rico-Lattes, Insulator semiconductor structures coated with biodegradable latexes as encapsulation matrix for urease, *Biosensors and Bioelectronics,* Vol. 20, 2005, pp. 2318-2323.

[7]. D. Brondani, C. W. Scheeren, J. Dupont, I. Cruz Vieira, Biosensor based on platinum nanoparticles dispersed in ionic liquid and laccase for determination of adrenaline, *Sensors and Actuators B,* Vol. 140, 2009, pp. 252-259.

[8]. L. I. B. Silvaa, F. D. P. Ferreira, A. C. Freitas, T. A. P. Rocha-Santos, A.C. Duarte, Optical fiber biosensor coupled to chromatographic separation for screening of dopamine, norepinephrine and epinephrine in human urine and plasma, *Talanta*, Vol. 80, 2009, pp. 853-857.

[9]. D. M. Sullivan, J. L. Havlin, Flow injection analysis of urea nitrogen in soil extracts, *Soil Sci. Soc. Am. J,* Vol. 55, 1991, pp. 109-113.

[10]. J. Gonzalez-Rodriguez, P. Perez-Juan, M. D.L. de Castro, Method for monitoring urea and ammonia in wine and must by flow injection–pervaporation, *Anal. Chim. Acta*., Vol. 471, 2002, pp. 105-111.

[11]. J. L. F. C. Lima, C. Delerue-Matos, M. C. V. F. Vaz, J. Agric, Flow injection system with potentiometric detection for the determination of urea content in milks, *Food Chem*. Vol. 46, 1998, pp. 1386-1389.

[12]. X. C. Hu, M. Takenaka, M. Kitano, H. Bandow, Y. Maeda, M. Hattori, Determination of trace amounts of urea by using flow injection with chemiluminescence detection, *Analyst,* Vol. 119, 1994, pp. 1829-1833.

[13]. D. Chirizzi, C. Malitesta, Potentiometric urea biosensor based on urease immobilized by an electrosynthesized poly(o-phenylenediamine) film with buffering capabi, *Sensors and Actuators B* , Vol. 157, 2011, pp. 211-215.

[14]. Y. Velichkovaa, Y. Ivanova, I. Marinova, R. Rameshb, N. R. Kaminib, N. Dimchevac, E. Horozovac, T. Godjevargovaa, Amperometric electrode for determination of urea using electrodeposited rhodium and immobilized urease, *Journal of Molecular Catalysis B*, Vol. 69, 2011, pp. 168-175.

[15]. W. O. Ho, S. Krause, C. J. McNeil, J. A. Pritchard, R. D. Armstrong, D. Athey, K. Rawson, Electrochemical sensor for measurement of urea and creatinine in serum based on ac impedance measurement of enzyme-catalyzed polymer transformation, *Anal. Chem.*, Vol. 71, 1999, pp. 1940-1946.

[16]. A. V. Rebriiev, N. F. Starodub, Enzymatic biosensor based on the ISFET and photopolymeric membrane for the determination of urea, *Electroanalysis,* Vol. 16, 2004, pp. 1891-1895.

[17]. L. Wei, J. Shih, Fullerene-cryptand, Fullerene-cryptand coated piezoelectric crystal urea sensor

based on urease, *Analytica Chimica Acta,* Vol. 437, 2001, pp. 77-85.
[18]. B. Kovàc, G. Nagy, R. Dombi, K. Toth, Optical biosensor for urea with improved response time, *Biosensors and Bioelectronics,* Vol. 18, 2003, pp. 111-118.
[19]. A. S. Jdanova, S. Poyard, A. P. Soldatkin, N. Jaffrezic-Renault, C. Marteiet, Conductometric urea sensor: use of additional membranes for the improvement of its analytical characteristics, *Analylica Chimica Acta*., Vol. 321, 1996, pp. 35-40.
[20]. B. Xie, B. Danielsson, An Integrated Thermal Biosensor Array for Multianalyte Determination Demonstrated with Glucose, Urea and Penicillin, *Analytical Letters*, Vol. 29, 1996, pp. 1921-1932.
[21]. P. Pookaiyaudom, P. Seelanan, F. J. Lidgey, K. Hayatleh, C. Toumazou, Measurement of urea, creatinine and urea to creatinine ratio using enzyme based chemical current conveyor (CCCII+), *Sensors and Actuators B,* Vol. 153, 2011, pp. 453-459.
[22]. G. A. Splittgerber, The catalytic function of enzymes, *J. Chemical Education*, Vol. 62, 1985, pp. 1008-1012.
[23]. T. C. Huang, D. H. Cheng, Variations of ammonium ion concentration and solution pH during the hydrolysis of urea by urease, *J. Chem. Technol. Biotechnol,* Vol. 55, 1992, pp. 45-51.
[24]. B. Sahoo, S. K. Sahu, P. Pramanik, A novel method for the immobilization of urease on phosphonate grafted iron oxide nanoparticle, *Journal of Molecular Catalysis B: Enzymatic,* Vol. 69, 2011, pp. 95-102.
[25]. A. Erdamar, F. Ayhan, İ.C. Koçum, H. Ayhan, Urease Immobilized Piezoelectric Quartz Crystal for Urea, *Conversion J. Biol. Chem,* Vol. 36, 2008, pp. 173-180.
[26]. R. P. Pogorilyi, V. P. Goncharik, L. I. Kozhara, Y. L. Zub, Covalent immobilization of urease on polysiloxane matrices containing 3-aminopropyl and 3-mercaptopropyl groups, *Applied Biochemistry and Microbiology,* Vol. 44, 2008, pp. 561-565.
[27]. A. F. S. A. Habeeb, R. Hiramoto, Reaction of proteins with glutaraldehyde Arch, *Biochem Biophys.,* Vol. 126, 1968, pp. 16-26.
[28]. L. H. Dubois, R. G. Nuzzo, Annu, Synthesis, structure, and properties of model organic-surfaces *Rev. Phys. Chem,* Vol. 43, 1992, pp. 437-463.
[29]. J. P. Folkers, J. A. Zerkowski, P. E. Laibinis, C. T. Seto, G. M. Whitesides, Designing Ordered Molecular Arrays in Two and Three Dimensions, *ACS Sym. Ser.* Vol. 499, 1992, pp. 10-23.
[30]. R. P. Pogorilyi, V. P. Goncharik, L. I. Kozhara, Yu. L. Zub, Covalent immobilization of urease on polysiloxane matrices containing 3-aminopropyl and 3-mercaptopropyl groups, *Applied Biochemistry and Microbiology,* Vol. 44, 2008, pp. 561-665.
[31]. X. W. Liu, J. P. Xiong, Y. W. Lv, Study on corrosion electrochemical behavior of several different coating systems by EIS, *Progress Organic Coatings,* Vol. 64, 2009, pp. 497-503.
[32]. D. I. Hay, R. L. Hartlfzj, Studies on the glycolytic-enghancing propertiesof human saliva, *Arch. Oral Bid.*, Vol. 11, 1966, pp. 337-347.
[33]. H. Barhoumi, A. Maaref, N. Jaffrezic-Renault, Experimental Study of Thermodynamic Surface Characteristics and pH Sensitivity of Silicon Dioxide and Silicon Nitride, *Journal of Langmuir,* Vol. 26, 2010, pp. 7165-7173.
[34]. C. Lages, E. Méndez. C. Lages, E. Méndez, Contact angle measurements under thermodynamic equilibrium conditions, *Anal Bioanal Chem,* Vol. 388, 2007, pp. 1689-1692.