AMPEROMETRIC UREA BIOSENSOR BASED PLATINUM ELECTRODE MODIFIED WITH A COMPOSITE FILM

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A novel amperometric biosensor for the detection of urea was prepared by immobilizing urease on a modified electrode of type polymeric film/platinum substrate. The polymeric films were obtained by electropolymerization of the corresponding monomers in the presence of different additives. These polymeric films were utilized as conducting films for amperometric urea sensing after covalent immobilization of urease onto film polymeric-coated electrode. The electrochemical performance of the modified electrode was studied by cyclic voltammetry, cronoamperometry, and SEM. The immobilized urease on the polyaniline film surface exhibited an excellent electrocatalytic response toward reduction urea. Amperometric response was measured as a function of concentration of urea at a fixed potential, in this case the open circuit potential. The obtained sensor showed rapid response and good reversibility. The response time was less than 10 s, while the reversible time was about 5-15 s. The enzymatic electrode exhibited high sensivity, a wide linear range and low detection limit for electrochemical detection of urea.

INTRODUCTION

Urea is one of the final products of protein metabolism. Urea is an omnipresent compound present in blood and various organic fluids.

The urea concentration in the blood lies between 2.5-6.7 mM (15-40 mg/dL) while pathophysiological range covers 30-150 mM (180-900 mg/dL). The primary function of the kidneys is to remove wastes from the body. These may include the by-products of normal physiologic processes, drugs, and various toxins. When the kidneys malfunction, such substances begin to accumulate. Over time, progressive kidney failure can result in uremia.[1-2] On the other hand, urea can passes directly into the milk through diffusion. Therefore, milk is the second major biological sample for the study of urea concentration.[3] A periodic monitoring of urea in milk can be used to predict the state of animal's health and the protein requirement in its diet.[4] Besides milk, presence of urea in agricultural land as a pollutant due to excessive use fertilizers is also widely known. Various methods were used for the determination of urea. Amongst these methods, detection through electrochemical mode is highly adopted and versatile. This method involves the use of electrochemical urea biosensor. In the development of electrochemical urea biosensors, immobilization of urease over modified electrodes is the key parameter which decides the sensitivity and reproductibility of the sensor.

Therefore, devices developed based on biocatalyst "urease" to analyze urea also known as urea biosensors are of vital importance.[1-5] For the fabrication of the urea biosensor, urease is immobilized over a substrate, which can be a polymeric film electrodeposited on a metallic electrode. In a series of our previous papers, it was described the obtainment of different polymeric

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films, their mechanical and electrochemical characteristics as a function of chemical composition of synthesis solution, as a function of morphological structure and conditions for obtaining.[6-11] After urease was immobilized in polymer film, in this case in polyaniline film, the immobilized urease catalyzes the urea conversion into ammonium and bicarbonate ions based on enzyme substrate reaction. Many biosensors have been developed for the determination of urea in the biological samples namely spectrometry, potentiometry with application of pH sensitive electrode, conductometry, coulometry, amperometry and inductometry.[12-24]

Urea determination has been performed regularly in the medical field to study the proper functioning of the kidney. It can be stated say that the urea biosensors mainly are used in the medical field and also, in the food industry. The food industry has the requirement of a sensing system to accurately analyze dairy products during their manufacture and quality control. Urea biosensor is a valuable tool for monitoring the urea content of adulterated milk. Urea can stress the environment because it decomposes to ammonia, which is very toxic, and so it can pollute the streams and rivers into which it drains. So urea biosensor can be an economical tool to monitor the concentration of urea to be between limits allowed. The commercial biosensors that are available, suffer the drawbacks of high cost, complicated construction, and require extra electrodes to compensate for electrical interferences. Therefore, the development of cost effective and disposable biosensors for the detection of clinically important metabolites, such as urea, is a scientific matter of great importance.

In the present paper, a new polymeric film based on polyaniline was synthesized and employed as a new electron-mediating support material for fabricating urease-immobilized electrodes. For this purpose, the urease-immobilized electrodes were prepared using the electrodeposited polyaniline films of various thickness and application to detection of urea by amperometric method.

EXPERIMENTAL

The electrochemical polymerizations were carried out using a conventional three electrodes system. A platinum electrode and a saturated calomel electrode (SCE) were used as counter and reference electrode, respectively. The reference electrode was placed in a separate cell and was connected to the electrolytic cell via a salt bridge that ends as a Luggin capillary in the electrolytic cell. This arrangement helps in

reducing the ohmic resistance of the electrochemical system. The working electrode was made from a platinum disk. In this paper were used: aniline 99.5%, urease, urea, Ringer-Brown solution, sulphuric acid 98% which were purchased from Sigma-Aldrich or Fluka and all were of analytical grade. Bidistilled water was used for all sample preparations. Cyclic voltammetry and electrochemical impedance spectroscopy were used to investigate the electrochemical characteristics of the obtained modified electrodes. Electrochemical experiments were carried out with an automated model VoltaLab 40 potentiostat / galvanostat with EIS dynamic controlled by a personal computer.

All the following potentials reported in this work are against the SCE. Scanning electron microscopy (SEM) was used to compare the microstructures of the deposited films.
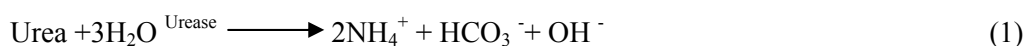
Preparation of the modified electrodes

The Pt electrode was carefully polished with aqueous slurries of fine alumina powder 0.05 μm on a polishing cloth until a mirror finish was obtained. After 10 min sonication, the electrodes were immersed in concentrated H_2SO_4, followed by thorough rinsing with water and ethanol. The prepared electrodes were dried and used for modification immediately. The pure PANI films were prepared from an aqueous solution of 0.2 mol/L aniline + 0.25 mol/L H_2SO_4 by cyclic voltammetry on the potential range of -250 mV to +900 mV with a scan rate of 10 mV/s and for a number of 20 cycles, when a thick film of PANI was electrodeposited. In this way, it was obtained PANI/platinum substrate modified electrode. Enzymatic electrode type PANI/Urease/Pt was obtained by dropping method. Aqueous urease solution of 0.76mg/mL was prepared using 38 mg urease (65.7 u.a./mg) and 50 mL bidistiled water in order to get the final enzyme layer casting solution 10 μL of the enzyme-layer casting solution was pipetted out onto the PANI/platinum substrate modified electrode surface and allowed to dry. We shall assume that the urease solution sipped into the porous PANI layer. Hence, we shall refer to the resulting enzyme biosensor by the full shorthand: PANI/UreaseDrop/Pt substrate (where Drop means that enzymatic electrode was obtained by dropping method). The biosensor thus made was always kept in the working buffer (in our case, the Ringer-Brown solution) at 4^0C when not in use, and rinsed with deionized water between experiments.

Then, we tried to obtain an enzymatic electrode type PANI/Urease/Pt by electropolymerization from a synthetic solution containing: 0.2 mol/L aniline + 0.25 mol/L H_2SO_4 + 0.76 g/L urease, by cyclic voltammetry on the potential scanning range of -250 to +900 mV at a scan rate of 10mV/s and for a cycles number of 20. Several attempts have been made but there was no co-deposition, consequently the enzymatic electrode was not obtained. New attempts were made and finally, the enzymatic electrode was obtained by co-deposition. But, in this case was obtained first a thin polyaniline film by electropolymerization from a synthetic solution containing: 0.2 mol/L aniline + 0.25 mol/L H_2SO_4, by cyclic voltammetry on the potential scanning range of -250 to +900 mV at a scan rate of 10mV/s and for a number of 10 cycles. The obtained modified electrode PANI/Pt was rinsed with bidistiled water and then it was immersed in another synthetic solution containing 0.2 mol/L aniline + 0.1 mol/L H_2SO_4 + 0.76 g/L urease. Using the cyclic voltammetry on the potential range of -250 to +900 at a scan rate of 10 mV/s and for 10 cycles, the enzymatic electrode PANI/UreaseCOD/Pt was successfully obtained. In this case we shall refer to the resulting enzyme biosensor by the full shorthand PANI/UreaseCOD/Pt, where COD is co-deposition.
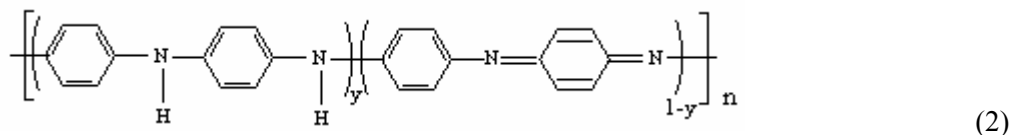
RESULTS AND DISCUSSION

Amperometry is the most commonly used technique for biosensors based on conducting polymers. Devices based on amperometry measure the change in current as a consequence of specific chemical reactions which take place at biotransducer electrode surface under nonequilibrium condition. The principle of the amperometry is based on the efficiency of the electron transfer between the biomolecule and underlying electrode surface in presence of electron mediator or conducting polymer. Moreover, amperometric biosensors are not only limited to the redox enzyme but also related to the biocatalyst reaction and interaction of the reaction product with conducting polymer to induce change in current.[15-16] Urea biosensor is the typical example of biocatalytic amperometric biosensor where ammonium ion which is a product of biocatalytic reaction interacts with polymer to induce a change in conductivity of the polymer. As mentioned before, in urea biosensors the enzyme immobilized to the electrode surface catalyzes the hydrolysis of the urea, in an overall reaction leading to the formation of ammonium, bicarbonate and hydroxide ions as shown below:

$$\text{Urea} + 3H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3^- + OH^- \tag{1}$$

The ionic products of the above reaction change the electronic properties of the biosensor electrode (modified with conducting polymers), which can be observed by various electrochemical techniques, in this case was used amperometric technique. Devices based on amperometry measure the change in current as a consequence of specific chemical reactions which take place at biotransducer electrode surface under nonequilibrium condition.

For the beginning, the enzymatic electrode type PANI/UreaseDrop/Pt was obtained by dropping method. In Fig. 1 are shown the polymerization cyclovoltammograms of aniline for obtainment of PANI/Pt modified electrode. Hence, these voltammograms were recorded during the growth of PANI film. As we can see from Fig. 1, at the cyclic potential sweep on the range -250 mV up to +900 mV, on the cyclic voltammograms appear three anodic oxidation peaks while, at the reverse potential sweep on the cathodic branch appears also three reduction peaks. This behaviour can be explained in the following mode: it is well known that polyaniline can exist in three different oxidation states such as leucoemeraldine (fully reduced form), emeraldine (partially oxidized form) and pernigraniline (fully oxidized form) as shown in the following scheme:

$$(2)$$

A very important characteristic of polyaniline consists in the fact that its structural units contain two different entities with different ponderables. Taking into account this property we can write thus: when $y = 1$, we have leucoemeraldine base, when $y = 0$ we obtained pernigraniline base and when $y = 0.5$ an intermediate state between leucoemeraldine and pernigraniline is obtained which is called emeraldine base. These forms of polyaniline are dependent on the applied potential. At the increasing anodic potential sweep the oxidation forms of polyaniline are obtained and on the anodic branch of the cyclovoltammogram appear the oxidation peaks while at the reverse potential sweep the reduction processes take place, on the cathodic branch of the cyclovoltammograms appear the reduction peaks. The three polyaniline oxidation forms correspond to the three anodic oxidation peaks while, the three polyaniline (PANI) reduction forms correspond to the three reduction peaks from the cathodic branch of cyclovoltammograms.[6-11]

On the platinum surface of the working electrode a polyaniline thick film was electrodeposited and for the obtainment of the enzymatic electrode, the urease was immobilized in the PANI thick film by the dropping method (see chapter intituled: Preparation of the modified electrodes).

Further, the obtained enzymatic electrode PANI/UreaseDrop/Pt was rinsed with bidistiled water and then immersed in a Ringer-Brown solution. Using the cronoamperometry method were registered the response currents at the addition of the urea samples. Hence, the testing of the obtained biosensor was carried out by amperometry method at the constant potential, in our case, at the open circuit potential.

In Fig. 2 is shown the variation diagram of response current at the successive additions of the urea samples.
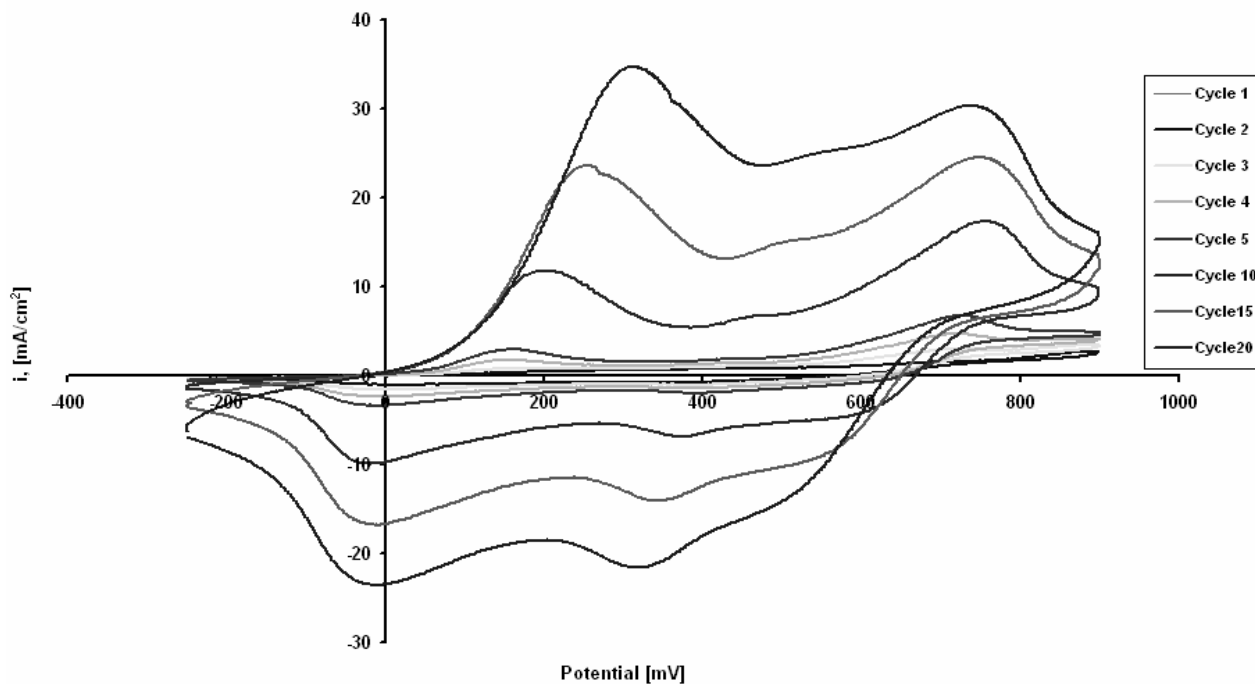
Fig. 1 – The obtainment of a PANI thick film by cyclic voltammetry from a synthesis solution of 0.2 M aniline + 0.25 M H_2SO_4 using a potential scan rate of 10mV/s, on the potential range of -250 mV up to +900 mV and for 20 cycles.
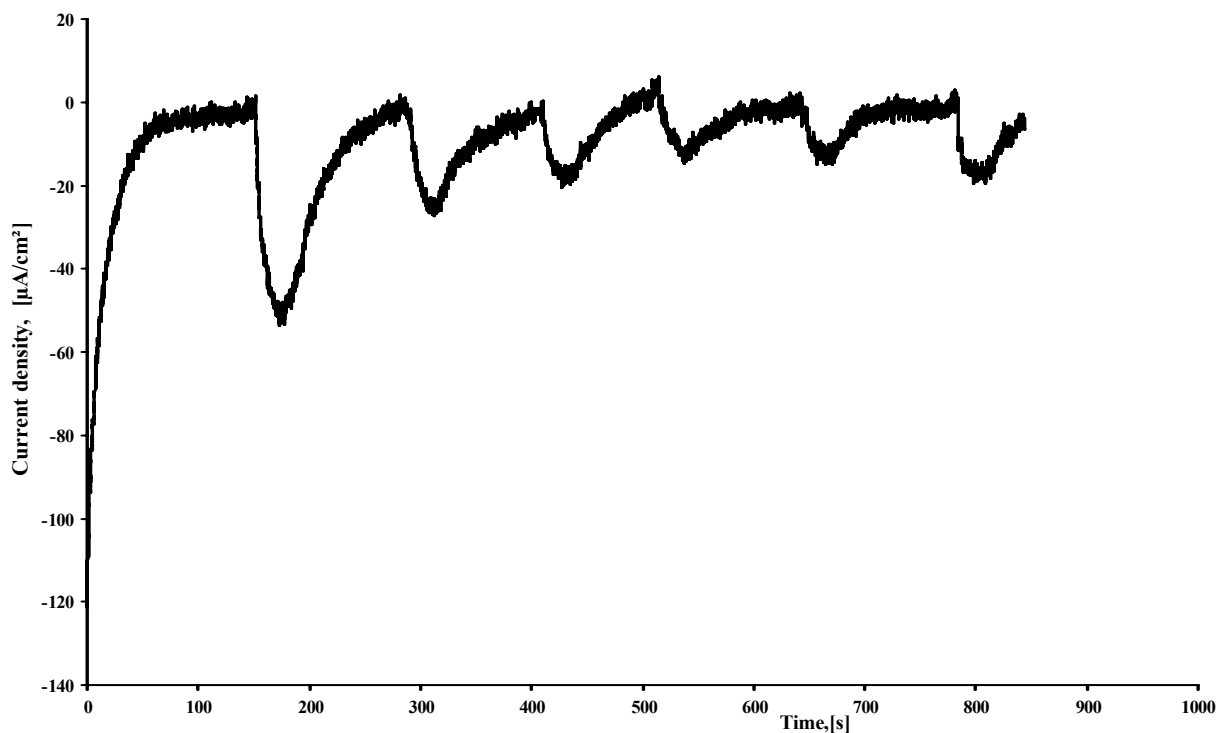

Fig. 2 – Response value of the peak current versus time at successive additions of the urea samples of 0.05 mmoli (1 ml urea solution 0.05 M).

Bellow, in Figs. 3 and 4 are presented the calibration curves of urea biosensor obtained by dropping method on different concentration ranges.

Analyzing the obtained results (see Figs. 2, 3 and 4), it can be observed that these results are not

satisfactory and for this reason it was fabricated a new urea biosensor namely PANI/UreaseCod/Pt – it was described in chapter intitulated: Preparation of the modified electrodes. As we shown, initially it was obtained a thin film of PANI (see Fig. 5) and

then the urea biosensor of type PANI/UreaseCod/Pt (see Fig. 6). For this reason, the obtained modified electrode type PANI thin film/Pt was immersed in a synthesis solution of 0.2 mol/L aniline + 0.1 mol/L H_2SO_4 + 0.76 g/L

urease, and then the electrode potential was scanned on a scanning range of -250 to +900 mV at a scan rate of 10mV/s and for a number of 10 cycles.
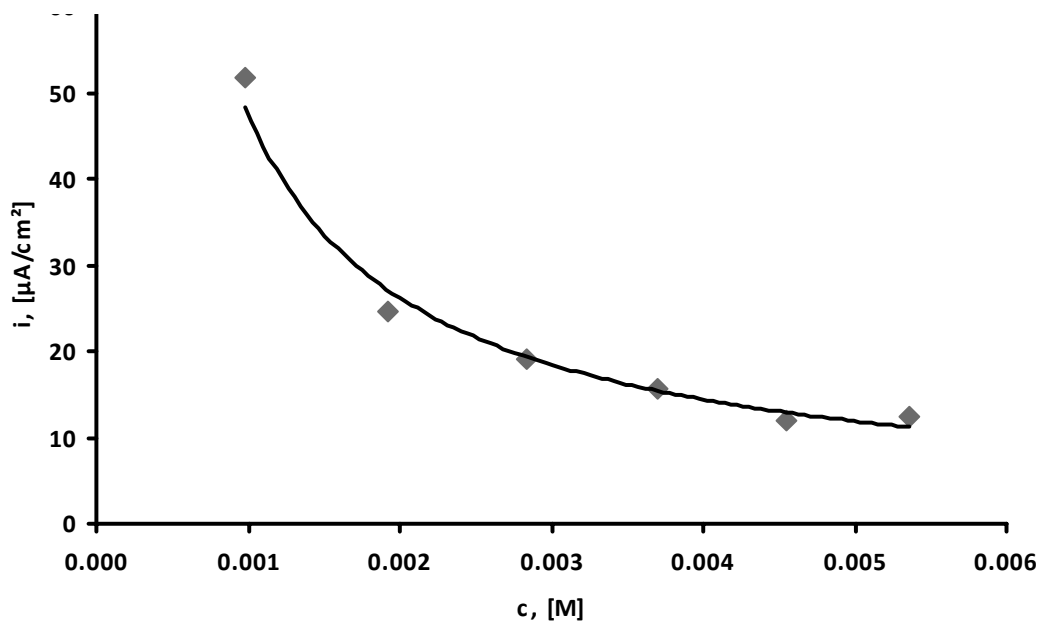

Fig. 3 – Calibration curve of urea biosensor type PANI/UreaseDrop/Pt namely, value of response current versus concentration of urea additions. The calibration curve has the following equation: $y = 0.1076x^{-0.8829}$.
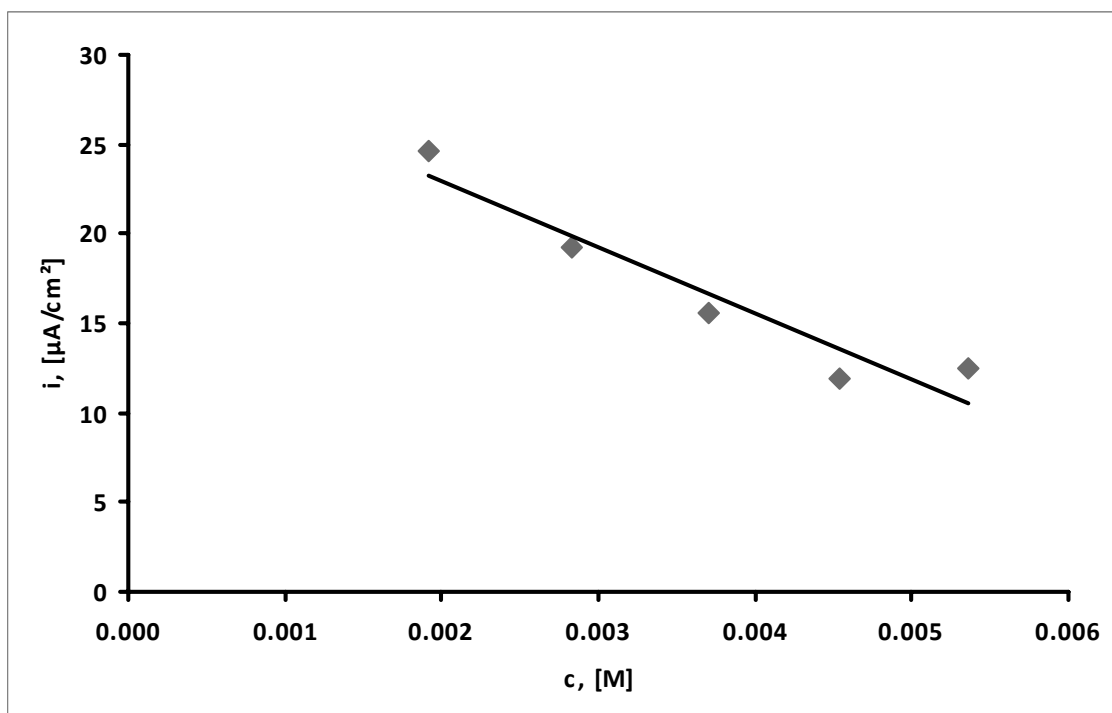
Fig. 4 – Linear range of variation of response current value versus concentration of urea additions for urea biosensor type PANI/UreaseDrop/Pt. Linear range of variation has the following equation: $y = -3702.7 x + 30.354$.
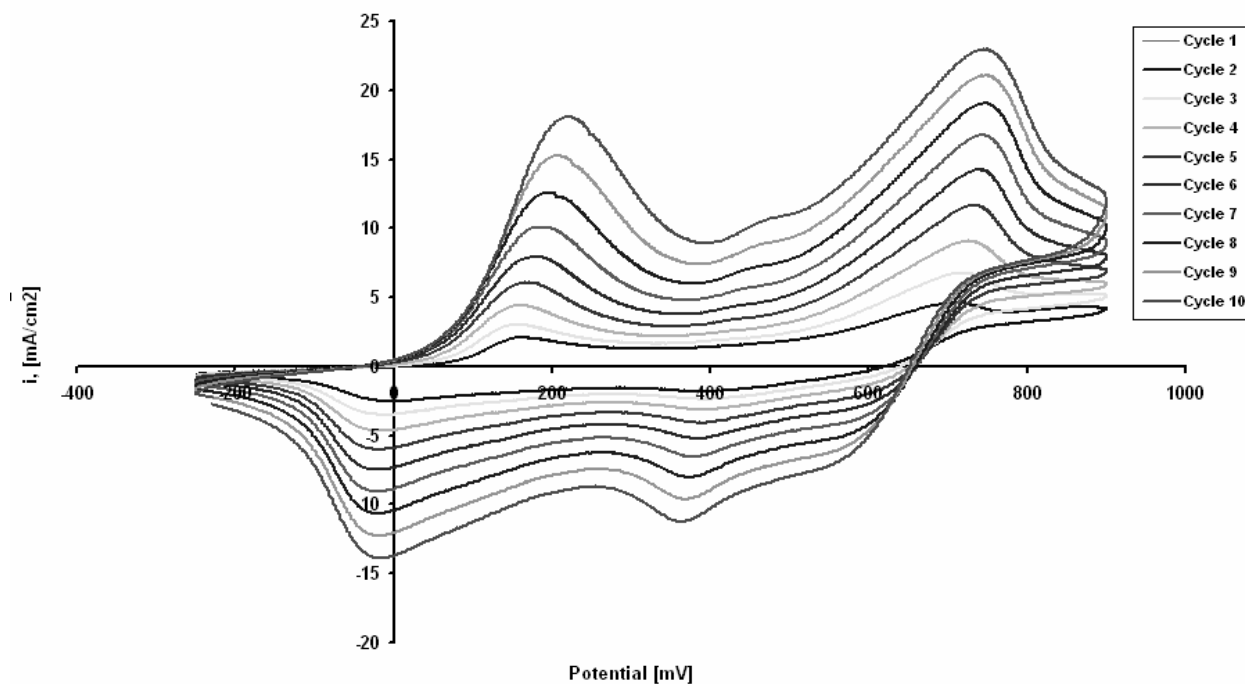
Fig. 5 – The obtainment of a PANI thin film by cyclic voltammetry from a synthesis solution of 0.2M aniline
+ 0.25 M H_2SO_4 using a potential scan rate of 10mV/s, on the potential range of -250 mV up to +900 mV and for 10 cycles.
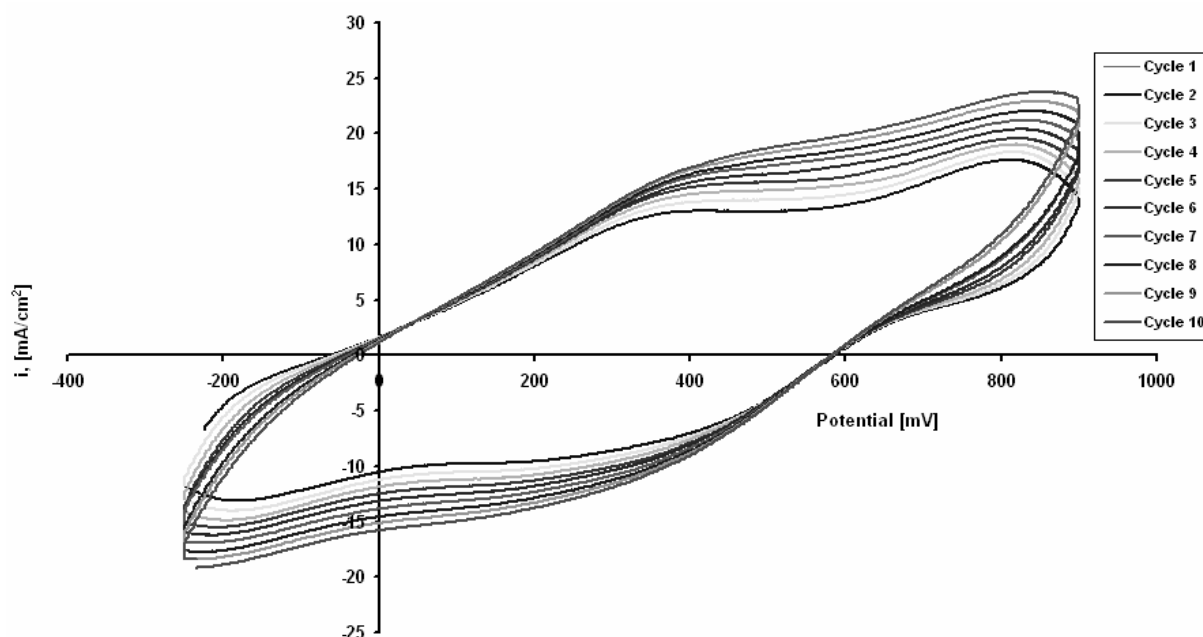

Fig. 6 – The recorded cyclovoltammograms at the urea biosensor obtainment by electropolymerization from a synthesis solution of
0.2 mol/L aniline + 0.1 mol/L H_2SO_4 + 0.76 g/L urease, on the potential scanning range of -250 to +900 mV at a scan rate of 10mV/s
and for a number of 10 cycles.

Analyzing Fig. 1 in comparison to Fig. 6, it can be observed that the shape of cyclovoltammograms from Fig. 6 differs very much comparatively to the shape of those from Fig. 1. This fact points out that,the redox processes are very different in the two electrdeposited films, respectively, in the thick PANI film (Fig. 1) and in the PANI/Urease co-electrodeposited film (Fig. 6). In the same time, this fact proves that the composition and morphological structure of the two electrodeposited films are different and that means that the urease was entrapped in polyaniline matrix. Thus, the urease enzymatic electrode type PANI/UreaseCOD/Pt was formed.

The testing of the obtained biosensor was carried out by amperometry method at the constant potential, in our case, at the open circuit potential, in a Ringer-Brown solution and the response currents were registered at the addition of the urea samples and after different times.

The calibration curve (response current density versus concentration) is presented in Fig. 8 and is described by the equation: $y = 2.9742x^{-0.4427}$
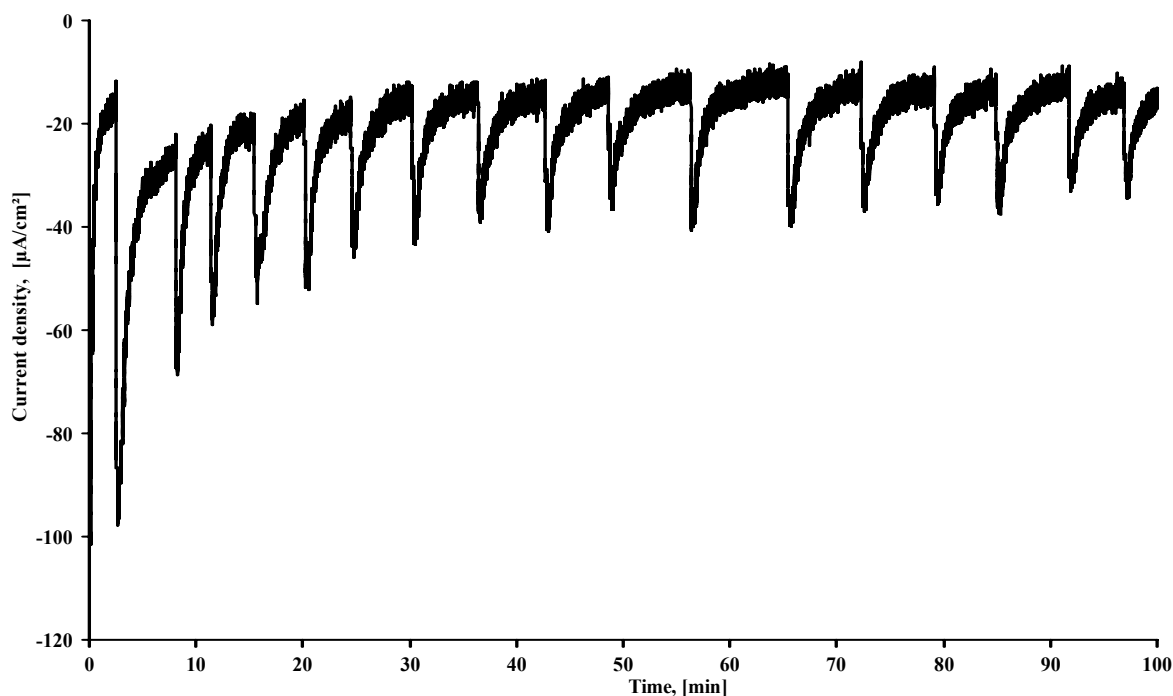

Fig. 7 – The response value of the peak current versus time at the successive additions of urea samples of 0.05 mmoli (1 mL solution urea 0.05 M), for urea biosensor type PANI/UreaseCod/Pt.
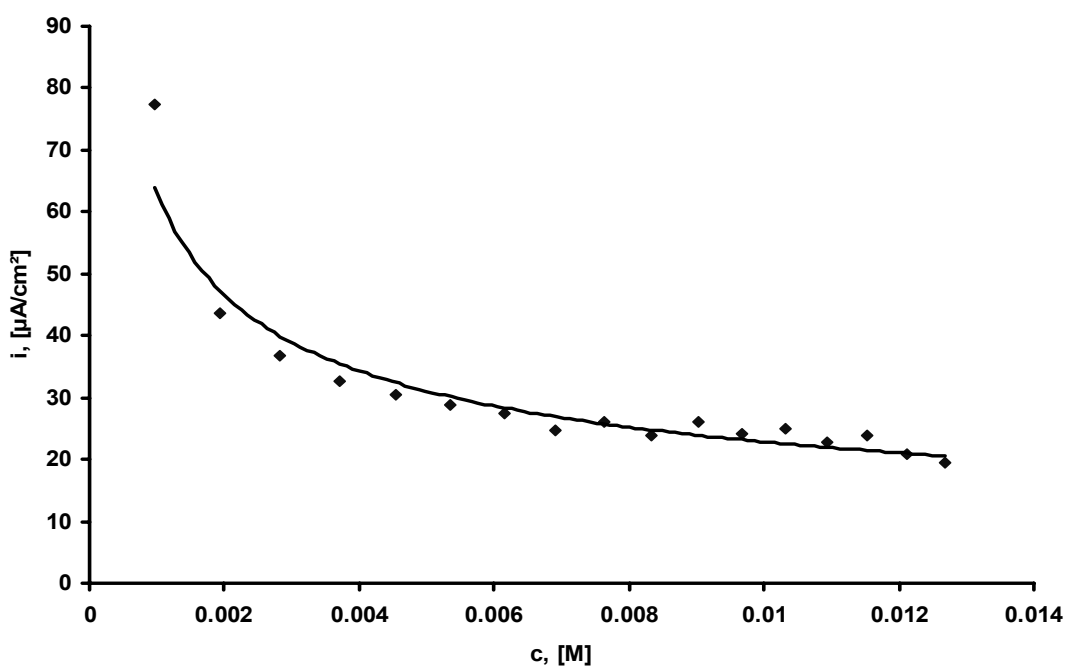

Fig. 8 – Calibration curve of urea biosensor of type PANI/UreaseCod/Pt which shows the variation of the response current versus urea concentration.
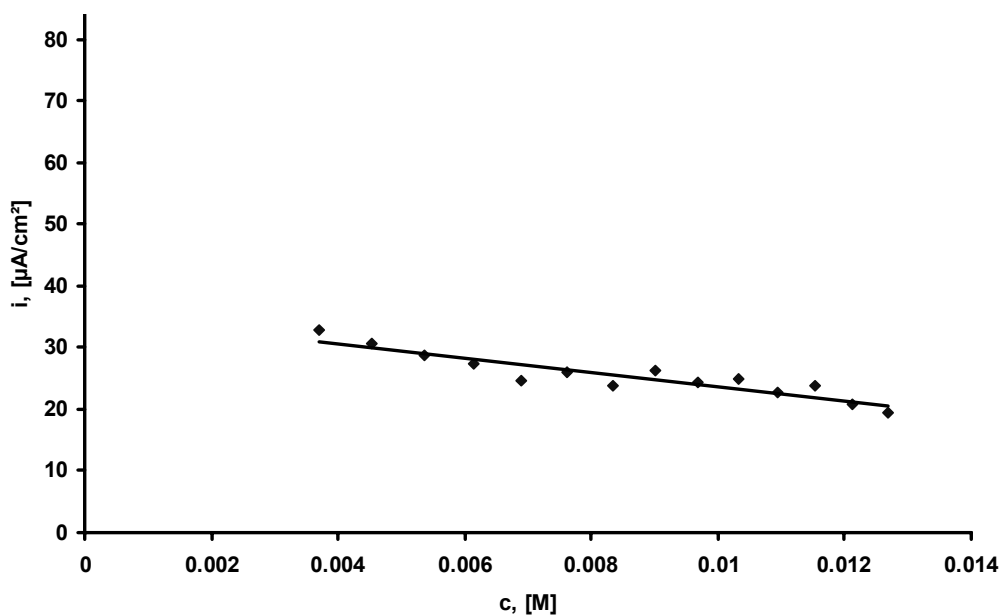
Fig. 9 – Linear response range of urea biosensor type: PANI/UreaseCod/Pt
Calibration equation of the right is: y = -1150.7 x + 35.206.

Further, the influence of testing time on the value of the response current was studied. In Fig. 10 is shown the variation diagram of the response current after 24 hours. As it can be observed, the activity of urea biosensor is still high enough and this fact proves that the sensor type PANI/UreaseCod/Pt is much better than the sensor type PANI/UreaseDrop/Pt. This fact points out that the obtainment of the enzymatic electrode by co-electrodeposition lead to much better results and to a much more stable and sensitive biosensor – see in comparison the Figs. 2, 7 and 10.
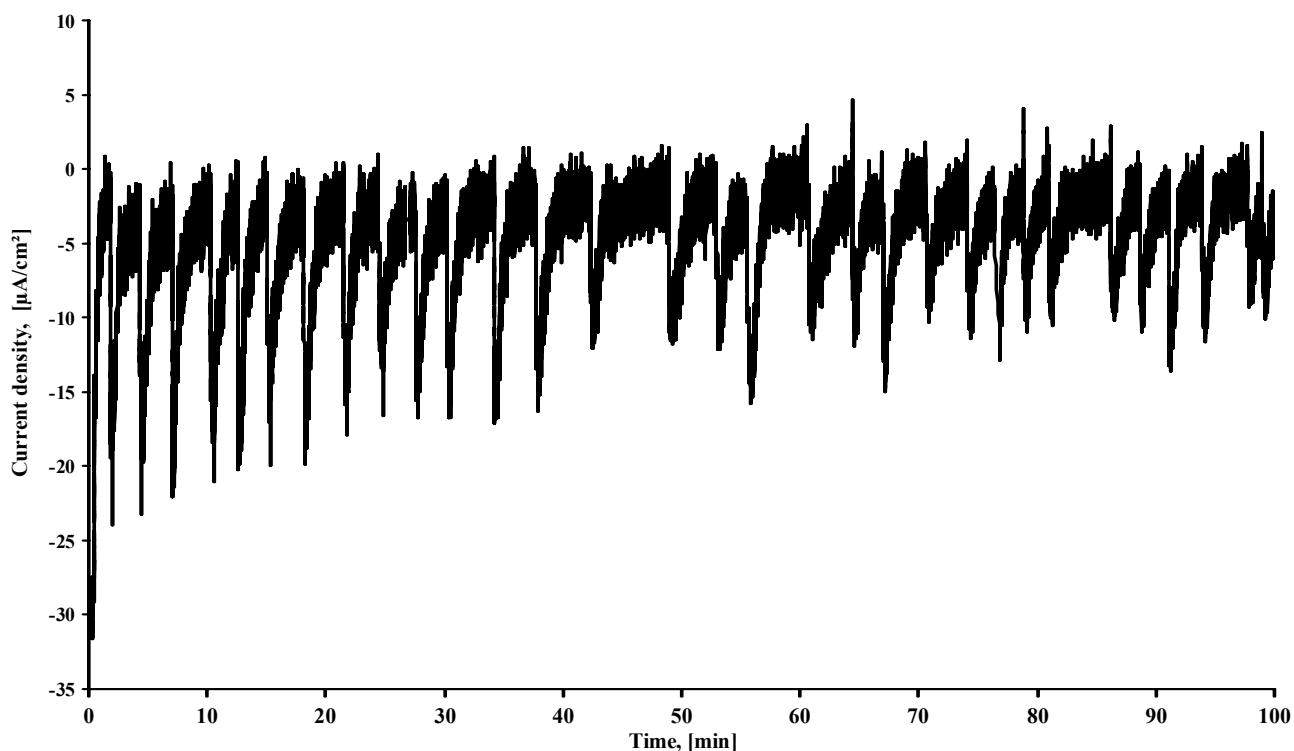

Fig. 10 – Response value of peak current versus time for urea biosensor type PANI/UreaseCod/Pt
to successive additions of urea samples of 0.05 mM (1 mL urea solution of 0.05 M) after 24 hours.
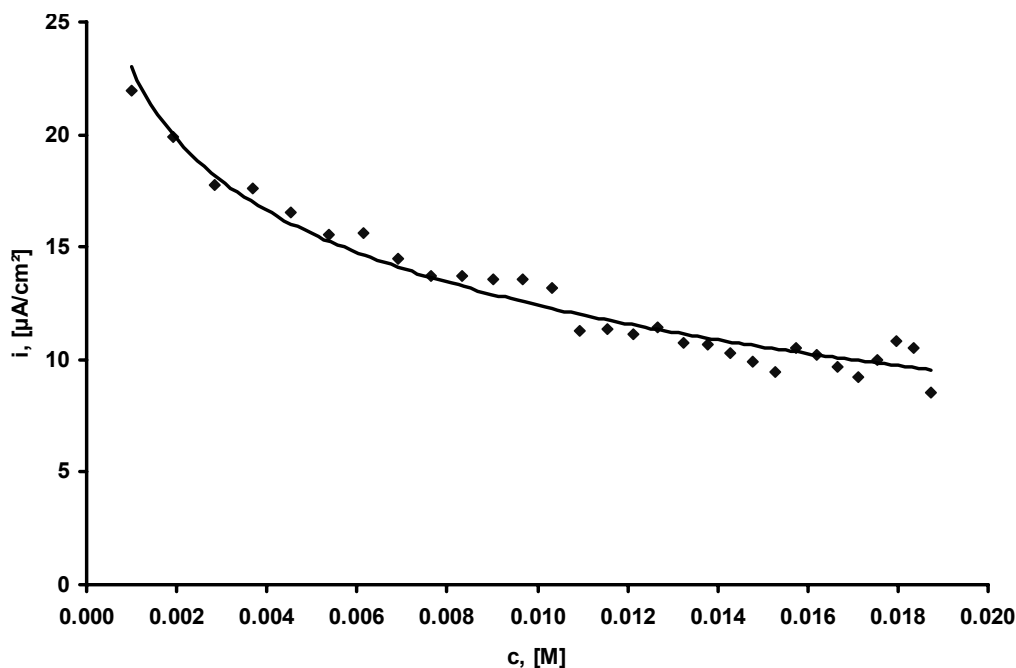
Fig. 11 – Calibration curve of urea biosensor of type PANI/UreaseCod/Pt which shows
the variation of the response current versus urea concentration.

Calibration curve from Fig. 11 is described by equation: y = -4,5778ln(x) - 8,6862.

In Fig. 12, it can be observed the response linear range of obtained urea biosensor type: PANI/UreaseCod/Pt.

Comparative analysis of current response values in Table 1 and Figs. 2, 7 and 10, shows that,

in the case of urea biosensor type PANI/UreaseCod/Pt the response currents are higher, so sensitivity is better and stability is much more higher than for biosensor type PANI / UreaseDrop / Pt. For this reason, is advisable to obtain the urea biosensors by co-electrodeposition of polyaniline and urease enzyme.
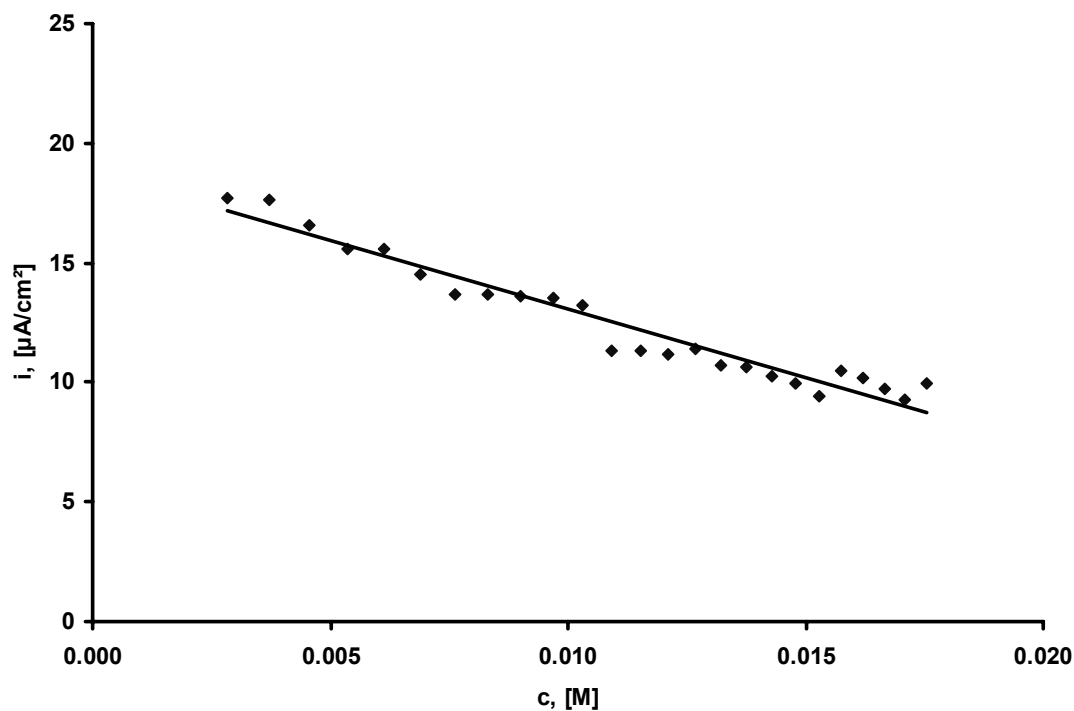

Fig. 12 – The response linear range for urea biosensor type: PANI/UreaseCod/Pt
Calibration equation of the right is: y =-571.81x + 18.768.

The values of response currents for urea biosensors synthesised in different modes, only for the first five samples
of urea added and different times

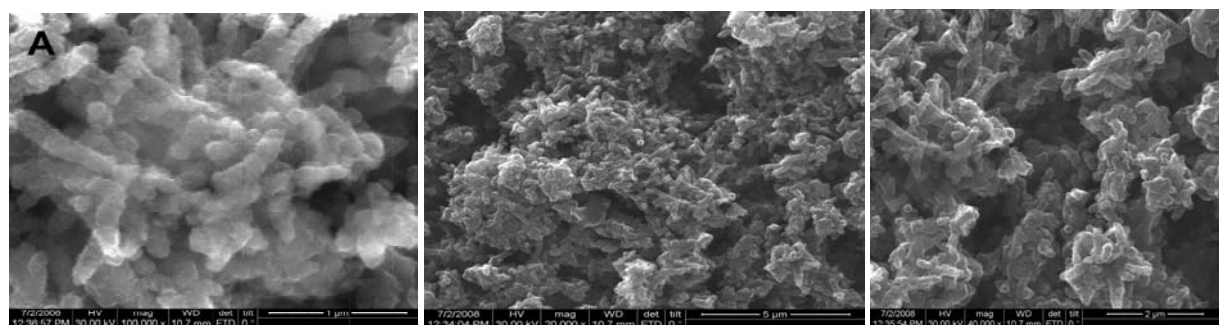| Enzymatic electrode	Response current peak			Response current peak (after 24 hours)				
	i_1, [μA/cm²]	i_2, [μA/cm²]	i_3, [μA/cm²]	I_1, [μA/cm²]	I_2, [μA/cm²]	I_3, [μA/cm²]	I_4, [μA/cm²]	I_5, [μA/cm²]
PANI/UreaseDrop/Pt	51.72	24.61	19.19				15.61	10.93
PANI/UreaseCod/Pt	77.44	43.68	36.65	19.89	17.74	21.97	32.72	30.57

Fig. 13 – SEM images of different magnitudes for polyaniline electrodeposited film.
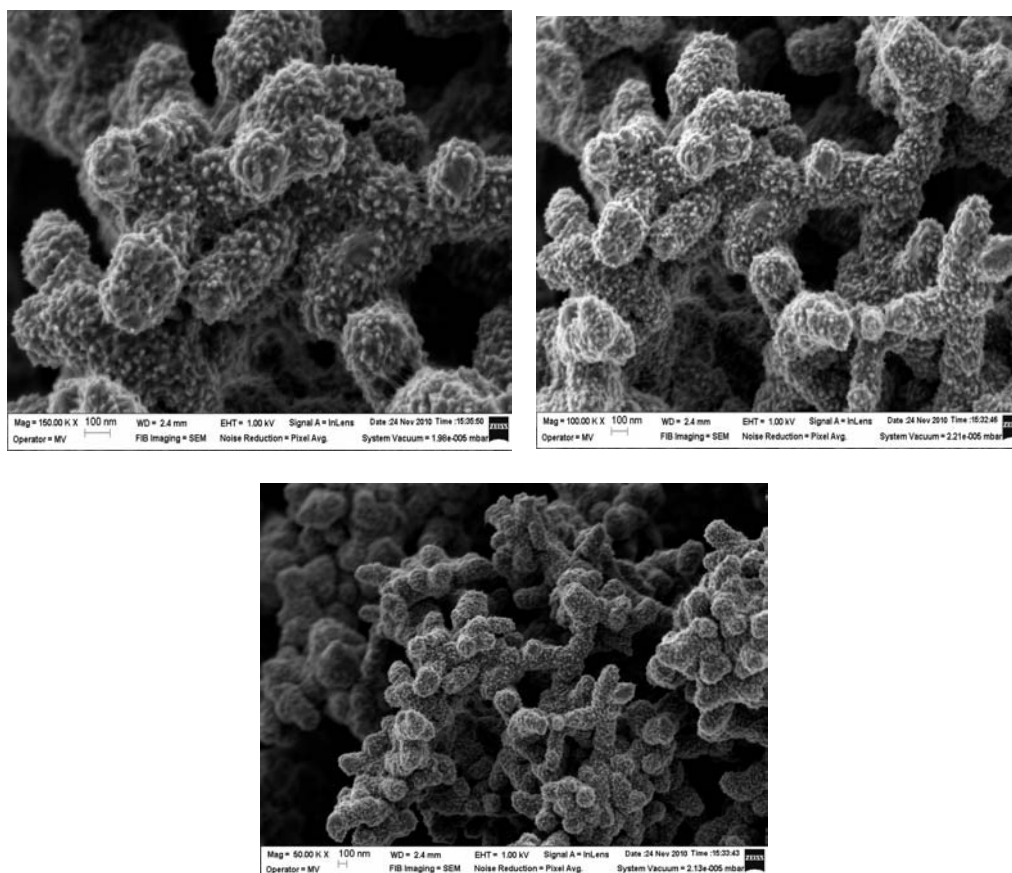

Fig. 14 – SEM images of different magnitudes for PANI/Urease co-electrodeposited film.

For a better understanding of these electrodeposited films behaviour were given more SEM images of different magnitudes – see Figs. 13-14.

Comparative analysis of the SEM images from Figs. 13 and 14 points out that the morphological structure of the two films differ greatly and also, one can see how the biomolecules of urease are entrapped into polyaniline matrix. In this way it can be explained the different behaviour of the two biosensors namely PANI/UreaseDrop/Pt and PANI/UreaseCod/Pt in the same conditions. Also, in the same mode the difference between the shapes of the cyclovoltammograms of the two electrodeposited films can be explained.

CONCLUSIONS

The presented result indicates that the electrochemical behaviour of polymer film electrodes is strongly dependent on the actual morphology of the polymer matrix.

The morphology of polymer matrix is an essential factor for the processes occurring in these films and in the design of electrodes for practical purposes.

The rate of charge transport, which is of vital importance in the use of these systems for electrocatalytic purposes, can be influenced by the temperature, the nature of the supporting electrolyte and its concentration.

The stability of the surface layer may be influenced by applying the proper conditions of film preparation, as well as high electrolyte concentration.

The obtained modified electrodes are stable, highly permeable for ions but at the same time fulfill the conditions needed for fast electron transfer.

REFERENCES

1. E. Karakus, S. Pekyardımc and E. Kılıc, *Artificial Cells, Blood Substitutes and Biotechnology,* **2005,** *33*, 329.
2. C. Eggenstein, M. Borchardt, C. Diekmann, B. Grundig, C. Dumschat, K. Cammann, M. Knoll and F. Spener, *Bioelectron.*, **1999,** *14,* 33.
3. M. J. R. Leema, S.M. V.Fernandes and A. O. S. Rangel, *J. Agric. Food Chem.*, **2004**, *52*, 6887.
4. G. Hof, M. D. Vervoorn, J. Lenaers and S. Tamminga, *J. Dairy Sci.*, **1997**, *80*, 3333.
5. A. V. Rebriiev and N. F. Starodub, *Electroanalysis,* **2004**, *16*, 1891.
6. V. Brânzoi, F. Brânzoi and L.Pilan, *Surface and Interface Analysis*, **2010**, *42*, 1266.
7. A. Prună, V. Brânzoi and F. Brânzoi, *Rev. Roum. Chim.*, **2010**, *55*, 293.
8. V. Brânzoi, F. Brânzoi and A.Muşina, *Rev. Roum. Chim.*, **2011**, *56*, 73.
9. A. Prună, V. Brânzoi and F. Brânzoi, *Journal Applied Electrochemistry*, **2011**, *41*, 77-81.
10. V. Brânzoi, A. Prună, and F. Brânzoi, *Molecular Crystal and Liquid Crystals*, **2008**, *485*, 853.
11. V. Brânzoi, F. Brânzoi and L. Pilan, *Materials Chemistry and Physics*, **2009**, *118*, 197.
12. D.Liu, K. Ge, K. Cheng, L. Nie and S.Yao, *Anal. Chim. Acta*, **1995**, *307,* 61.
13. M. Mascini, *Sens. Actuators B*, **1995**, *29*, 121.
14. X. Xie, A. A. Suleiman and G. G. Guilbault, *Talanta,* **1991**, *38*, 1197.
15. S. B. Adeloju, S. J. Shaw and G. G. Wallace, *Anal. Chim. Acta,* **1993**, 281, 611
16. S. B. Adeloju, S. J. Shaw and G. G. Wallace, *Anal. Chim. Acta*, **1993**, *281*, 621.
17. M. F. Esteve and S. Alegret, *J. Chem. Educ.*, **1994**, *71,* A67.
18. D. Liu, M. E. Meyerhoff, H. D. Goldberg and R.Brown, *Anal. Chim. Acta*, **1993**, *274*, 37.
19. K. Chen, D. Liu, L.Nie and S. Yao, *Talanta*, **1994**, *41*, 2195.
20. A. S. Jdanova, S. Poyard, A. P. Soldatkin, N. Jaffrezic-Renault and C. Martelet, *Anal. Chim. Acta,* **1996**, *321*, 35.
21. H. Sangodkar, S. Sukeerthi and R. S. Srinivasa, *Anal. Chem.***,** **1996**, *68*, 779.
22. R. E. Adams and P. W. Carr, *Anal. Chem.*, **1978**, *50,* 944.
23. S. B. Adelju, S. J. Shaw and G. G. Wallace, *Anal. Chim. Acta*, **1996**, *323*, 107.
24. W. O. Ho, S. Krause, C. J. McNeil, J. A. Pritchard, R. D. Armstrong, D. Athey and K. Rawson, *Anal. Chem.*, **1999**, *71*, 1940.