Scientific report

regarding the project implementation in the period January-December 2012

During this year were carried out the activities included the work plan for the accomplishment of general objective of the project, an electronic system based on chemical sensors and biosensors for analysis of biogenic amines. In the following pages will be describe the activities carried out, objectives achieved and dissemination activities carried out in this first year of the project.

1. Modeling and simulating studies of interaction between biogenic amines and receptors of sensors and biosensors were carried out by using HyperChem and Matlab software, respectively. From the modeling studies was determined the mechanism of interaction between biogenic amines and sensitive compounds, in the case of sensors or enzymes from the receptor element, in the case of biosensors.

Amine oxidases interact with biogenic amines by means of metal ions located in the active center of the enzyme cleaving amino groups. From the enzymatic reaction results a carbonyl compound, ammonia and H_2O_2. Monoamine oxidase and diamine oxidase have a high selectivity, this fact being related to the nature and chemical structure of the active center and to conformation of the protein chain.

When the enzyme is putrescine oxidase, the biocatalytic reaction takes place by the interaction of the amino group of putrescine and the active center of the enzyme resulting an aldehyde-amine compound, ammonia and H_2O_2. H_2O_2 is detected by electrochemical oxidation on the biosensor surface by applying a suitable potential, which depends on the nature of the electrode and on the presence of electron mediators.

In the case of horseradish peroxidase, an enzyme which have the active center outside of the protein molecule, the interaction does not occur directly with the amine molecule but by H_2O_2 generated from the action of an amine oxidase (e.g. diamine oxidase) by means of the Fe (III) ion located in the active center of the enzyme. Therefore, the peroxidase can be used for the construction of bi-enzymatic biosensors containing an amine oxidase and peroxidase.

Tyrosinase can be used as a biocatalyst for the detection of biogenic amines containing phenolic groups in the molecule. The mechanism of interaction is different for monophenols (e.g. tyramine) and diphenols (e.g. dopamine). Interaction occurs between phenolic group from the molecule and the active center of the enzyme that contains two Cu ions. The catalytic reaction occurs in two steps; in the first stage take place the hydroxylation the ortho position (for monophenols) followed by oxidation of diphenols to o-quinone. Ortho-quinone is electrochemically reduced at a potential depending on the nature of the electrode and electron transfer mediators.

In all cases studied by modeling, the biosensor detection mechanism is governed by the transfer of electrons (the slowest step) and applying a potential leads to an acceleration of the reaction due to electrochemical transformation of reaction products resulted from enzymatic reaction.

The simulations using Matlab software were performed considering that the electrochemical biosensor has a flat geometry, the enzyme layer is deposited on the surface and it is covered with a semipermeable membrane to ions, reagents and reaction products. It was determined the effect of pH and temperature on the biosensor response. On the other hand, the influence of limiting factor on reaction rate, diffusion or transfer of electrons, on the range of linearity (linear dependence of biosensor response and analyte concentration) and response time of the biosensor were studied. It has been shown that diffusion has a huge influence on the range of linearity (variations of the order of magnitude) and a lower influence on the biosensor response. If the rate limiting step is the transfer of electrons influence the linearity range is reduced while response times vary significantly.

From the results presented here it can be concluded that in these studies was determined the mechanism of interaction between biogenic amines and receptor element of sensor or biosensor by modeling and simulating studies. In addition, the influence of limiting factor of reaction rate, pH and temperature on linearity and time response of the biosensor during amperometric or potentiometric measurements were quantified.

2. Selection of sensors and biosensors design

It was carried out a market study in order to choose among commercially available electrodes that are optimal for the purposes of this project. Thus, for small amounts of sample was choose the screen-printed sensors based on different materials from Dropsens. For larger amounts of sample were acquired carbon electrodes in the shape of wire. Also, it were designed and constructed novel sensor designs. Furthermore, it were constructed electrodes in the form of platinum disk, carbon paste electrodes, ITO electrodes, screen-printed electrodes from Au in the form of sensor arrays, all of them with adequate size and low cost. In the case of carbon paste electrodes was changed the chemical composition of carbon paste by using different materials based on carbon (graphite, carbon nanoparticles, carbon nanotubes, carbon nanofibres) and different electroactive materials (Lu, Gd and Dy bisphthalocyanines, Co-phthalocyanine, Fe-phthalocyanine, di-Litium phthalocyanine, ferrocene), which are sensitive to biogenic amines and can be electron transfer mediators, in the case of biosensors. From exploratory measurements using potentiometric and voltammetric techniques it was established that voltammetric methods are more appropriate because these present higher sensitivities. Equilibrium potential of sensors and biosensors in the analyzed sample will be used to establish correlations with other physico-chemical parameters of samples. It was established that the study of the electrochemical behavior of sensors and biosensors will be carried out using cyclic voltammetry. For increasing of sensitivity and peaks resolution square wave voltammetry will be used. For routine measurements chronoamperometry will be used applying optimum potential for oxidation or reduction of target compound or a product obtained from the enzymatic reaction.

In conclusion, it was chose and constructed appropriate designs of sensors and biosensors, the system can be adapted depending on the amount of sample available and on the physico-chemical properties of sample.

3. Improvement of sensors qualities and design of novel sensors and biosensors

The activities carried out were aimed to develop the design of novel sensors and biosensors by chemical and biochemical modification of commercial electrodes or electrodes constructed in the laboratory. Modifier materials were purchased after a rigorous market study. Some of these commercial materials (Co-phthalocyanine, pyrrole, aniline, etc.) were purified by recrystallization or distillation. For electrochemical or chemical synthesis of other sensitive materials reagents and solvents required were purchased. As in the case of commercial materials it was necessary an advanced purification because the presence of impurities in the sensitive materials can decisively influence the sensors or biosensors characteristics.

3.1. Synthesis and characterization of molecular materials

For the construction of sensors and biosensors are required sensitive materials with adequate properties that are able to provide a measurable response when these interacting with the analyte. Based on the experience in this field, it were synthesized a series of coordinative compounds. Bisphthalocyanines of some lanthanide ions (Lu, Gd, Dy) were synthetized using a method that does not use solvents. Thus, appropriate amounts of lanthanide acetate and phthalonitrile are mixed in the solid state and then heated and maintained at a temperature of 250°C for 3 hours. As a result of the reaction it was obtained a blue-green solid (a mixture of the neutral form and the reduced form of the bisphthalocyanine). The solid was cooled, solved in chloroform and passed through a chromatographic column containing neutral Al_2O_3 and using $CHCl_3$ as carrier solvent in order to separate the neutral form of bisphthalocyanine ("green" form). Separation was monitored by using thin layer chromatography and UV-Vis spectroscopy. The raw product was purified by recrystallization from heptane obtaining a green solid (reaction yield is near to 25% for all three bis-phthalocyanines synthetized). Bisphthalocyanines obtained were characterized by UV-Vis, NIR and FTIR demonstrating their purity and presence of characteristic peaks in UV-Vis, NIR and FTIR spectra. Therefore, it were obtained, purified and physico-chemical characterized several compounds that will be tested as sensitive materials for detection of biogenic amines.

3.2. Synthesis and characterization of conducting polymers

For the preparation of sensors based on conducting polymers were used the following monomers: pyrrole, aniline and 3-methylthiophene. As doping agents were used several compounds that allow obtaining polymer films with morphologies, sensitivities and different redox properties. Thus, for the electrochemical synthesis of polyaniline were used: HCl, HNO_3, H_3PO_4, CH_3COOH, $HClO_4$ and $H_2C_2O_4$. For the synthesis of polypyrrole were used $K_4[Fe(CN)_6]$, $Na_2[Fe(CN)_5NO]$, $H_3PW_{12}O_{40}$, H_2SO_4, Na_2MoO_4, sodium salt of 9,10-

anthraquinon-2-sulfonic acid acid, sodium dodecansulfonat, sodium decansulfonat, p-toluenesulfonic acid acid and phosphate buffer of pH 7. In the case of poly-3-methylthiophene doping agents used were: $LiClO_4$, $LiCF_3SO_3$, tetrabutylammonium perchlorate and tetrabutylammonium tetrafluoroborate.

From the solution containing monomer and doping agent were synthesized polymer films with different properties using different electrochemical techniques such as chronoamperometry, chronopotentiometry, cyclic voltammetry and square wave voltammetry. It was shown that by using chronoamperometry the polymeric films obtained are optimal for use in the development of sensors and biosensors. This technique allows a strict control of layer thickness and over-oxidation degree.

Synthetized polymers were characterized by IR spectroscopy in order to determine the degree of over-oxidation. From the analysis of IR spectra were determined the optimal conditions of polymerization so that the polymer is not over-oxidated. A maximum potential of 0.8V used for electrochemical synthesis ensures a very low degree of polymer over-oxidation.

The morphology of polymer films was determined by scanning electron microscopy (SEM) and atomic force microscopy (AFM). These studies were carried out during the research stage at the University of Valladolid (Spain). It were shown that the parameters having a major influence on the morphology of the polymeric films are the chemical nature of doping agent and the electrochemical technique used for synthesis.

In conclusion, conducting polymers were synthesized and characterized spectroscopically and microscopically. From the spectroscopic, microscopic and electrochemical studies were demonstrated that polypyrrole is most suitable for the construction of sensors and biosensors, due to the compatibility with biogenic amines and to the double role of electron mediator and immobilization matrix, in the case of biosensors. Poly-3-methylthiophene has the disadvantage that the electrochemical synthesis is carried out only on acetonitrile solution, increasing the manufacture costs. Polyaniline was synthesized and presents suitable electrochemical properties only in strongly acidic solutions, conditions that cannot be used for enzymes.

3.3. Fabrication of novel sensors and biosensors

For the fabrication of sensors and biosensors different methods were used that have as objective deposition of sensitive material on a solid support, with a particular design, suitable for electrochemical measurements.

a) The choice of substrates was made based on nature of sensitive material and the optimal method of deposition. For this purpose Pt wire was purchased and electrodes were fabricated in the shape of disk. Other substrates used for fabrication of electrodes were ITO (indium tin oxide) coated glasses, useful for electrochromic measurements. Also, it were used screen-printed gold electrodes in the configuration of a sensor array. It were purchased and used different screen printed electrodes containing the same device the working electrode, the counter and the reference electrode. The materials used in construction of these screen printed electrodes are carbon (C), carbon nanotubes, carbon nanofibers, graphene, cobalt-phthalocyanine-C, Prussian blue-C, platinum, C-platinum nanoparticles and Au. For electrochromic measurements were acquired optical-transparent screen printed electrodes based on ITO. Another type of electrodes used were C wire electrodes. Electrode area was among $0.785mm^2$ and $12.56mm^2$ for disk electrodes, $1cm^2$ of ITO electrodes and $52.5mm^2$ for wire electrodes.

The electrochemical characteristics of all electrodes were studied. It were shown that some of them can be used as voltammetric sensors without any changes. In other cases modifying of them with other sensitive materials, commercial or synthesized in the laboratory, was absolutely necessary. For the construction of biosensors was necessary the modification of receptor element with enzyme.

b) Depending on the size of the substrate and the amount of material available several methods for deposition of sensitive layer were used.

b.1. In the case of sensors based on conducting polymers electrodeposition technique (electrosynthesis) from a solution containing the monomer and doping agent was used. The solvent was water in the case of polyaniline and polypyrrole and acetonitrile in the case of poly-3-methylthiophene. The optimal electrochemical technique for electrosynthesis was chronoamperometry, which ensure an uniform deposition, a short deposition time (30-120s) and a control of over-oxidation process of polymer. Furthermore, this technique controls very precise of electric charge used on electrosynthesis and it can be accurately calculate the thickness of the deposited polymer. It were electrosynthetized polymeric films

with thicknesses between 200 nm and 50μm. Optimal thickness of polymer for the fabrication of sensors is between 2 and 10μm, thickness that provides mechanical stability and good sensitivity. Also, this thickness is ideal for immobilizing the enzyme in the case of biosensors.

b.2. In the case of other sensitive materials other deposition methods were used, described in the following paragraphs. The most advanced technique used is Langmuir-Blodgett technique (employed during the research stage at the University of Valladolid), technique providing the control of sensitive layer of sensor or biosensor, at molecular level. ITO substrate was used as substrate and sensitive materials were bis-phthalocyanines of Lu, Gd and Dy. In order to facilitate the deposition of nanostructured monolayers arachidic acid was used, also. The quality of monolayers was studied by BAM microscopy. When biosensors were fabricated, the enzyme was introduced in aqueous sub-phase (0.01M phosphate buffer and 0.1 M NaCl) and the mediator on the surface of the subphase. In the first step were recorded surface pressure isotherms determining the surface pressure where monomolecular layer has a high degree of order and can be transferred to the solid support. At the optimal surface pressure monolayers were transferred by immersion-emersion cycles, in a variable number of monolayers, between 10 and 30, depending on sensitive properties of materials.

For disk screen-printed electrodes cast or drop-and-dry deposition technique was used, both for deposition of sensitive material and enzyme, in the case of biosensors.

ITO substrate was used also in the case of layer-by-layer techniques (LBL) and spin-coating method.

For the fabrication of other sensors carbon paste electrodes technique was used, in which carbonaceous material (graphite, carbon nanotubes, C nanopowder) was mixed with mineral oil in a ratio that can ensure a good electrical conductivity and appropriate mechanical strength (the weight ratio is 1:1.3). For increased sensitivity sensitive substances or enzymes were used, which were deposited on the surface of carbon paste electrodes. In some cases, sensitive materials were placed within the carbon paste (for example, bis-phthalocyanines). The percentage of the phthalocyanine in relation with the carbon material is 15%.

b.3. Immobilization of enzymes on electrodes was achieved by several methods, namely: by physical adsorption, by retaining in solid matrix (carbon paste), by electropolymerization and by Langmuir Blodgett technique. In order to increase the stability of the enzyme layer, regardless of the method of deposition , it was used cross-linking process with glutaraldehyde. The enzymes employed were tyrosinase, peroxidase, mono- and diamine oxidase. Enzyme layer comprises between 100 and 300 enzyme units per biosensor.

In the case of electron mediators, these were deposited on the sensitive element following two strategies. The mediator and enzyme layers were deposited separately by the same technique or using different techniques. For example, over the film of polypyrrole obtained by electrochemical synthesis the enzyme was adsorbed and then cross-linking reaction is performed. Also, in this category is included the modification of carbon paste electrodes, metalic or screen-printed by enzyme adsorption followed by cross-linking.

When there was a physical and chemical compatibility between the enzyme and the mediator, mixed layers were deposited by an appropriate method. This is the case of bisphthalocyanines which were deposited together with enzyme by Langmuir-Blodgett technique, of polypyrrole electrosynthetised from a solution containing monomer, doping agent and enzyme or of carbon pastes made from carbonaceous material, mediator, enzyme, and conglomerate agent.

3.4. Sensors and biosensors prepared were characterized by spectroscopic (UV-Vis, NIR and IR) and microscopic (SEM, and AFM BAM) techniques.

From the analysis of UV-Vis, NIR and IR spectra was determined the order degree of molecules, relative molecular orientation to the solid substrate (perpendicular, parallel or under other angles), the formation of new covalent bonds, the existence of enzyme on biosensor receptor, etc. BAM (Brewster angle microscopy) allowed determining the morphology of monolayer before transferring on solid substrate. Morphology of receptor element was determined by SEM and AFM.

For the sensors and biosensors prepared by using Langmuir-Blodgett technique was determined that bisphthalocyanine molecules were oriented almost perpendicular to the substrate surface of ITO, the arachidic acid molecules were perpendicular to the surface and form a bi-layer and the enzyme molecules are retained in two-layer structures similar to cellular membranes. In addition, this biomimetism promotes

the enzymatic activity due to changing quaternary structure and accessibility of the active center for analyte molecules, as shown in the measurements carried out with this type of biosensors.
In the case of enzyme immobilization by using cross-linking reaction it were identified novel covalent bonds between enzyme molecules and between enzyme and immobilization matrix, e.g. polypyrrole. From measurements carried out with these biosensors was found that cross-linking leads to a decrease in enzymatic activity but also is an increasing of biosensors durability. This is related to the change in conformation of the enzyme. Therefore, there must be an equilibrium between sensitivity of biosensors and their durability.
Microscopic techniques were shown that mixed layers deposited by Langmuir-Blodgett technique have a very low roughness due to the homogeneity of monolayers transferred onto solid substrates. In the case of polypyrrole, morphology depends on the nature doping agent and the electrochemical technique used.
In addition to the originally proposed work plan it were carried out a series of studies on the determination of electroactive compounds in emulsions with the purposes of determining the capacity of sensors and biosensors to function in this type of environment. The results obtained with polypyrrole sensors were good and were published. This study was necessary because the sensors and biosensors will be used for the analysis of biogenic amines in foods with a minimal processing of samples, thus in complex heterogeneous environments. It was also studied the encapsulating of enzymes before immobilization in order to increase the sensitivity of biosensors.
Therefore, in this year were carried out all the activities included in the work plan obtaining novel sensors and biosensors, with new designs, from different sensitive materials deposited by means of nanotechnologies and characterized by spectroscopic and microscopic methods.

Dissemination of results

Dissemination of research results was carried out by publishing ISI papers, publication of a chapter in monograph and participation to international and national conferences.

Publishing of ISI papers

1. C. Apetrei, Novel method based on polypyrrole-modified sensors and Emulsions for the evaluation of bitterness in extra virgin olive oils, Food Research International 48 (2012) 673-680; doi: 10.1016 / j.foodres.2012.06.010, journal impact factor 3.15; relative influence score 2.47386.
2. I. M. Apetrei, C. Apetrei, Amperometric biosensor based on polypyrrole and tyrosinase for the detection of tyramine in food samples, Sensors & Actuators: B. Chemical, 2012, Major revision (11 November 2012), journal impact factor 3.898; relative influence score 1.85283.

Chapters in international monographs

1. C. Apetrei, M. Ghasemi-Varnamkhasti, Biosensors in food authentication PDO, Chapter 11, Protected Designation of Origin Food: Methodologies and Applications, Ed. A. Gonzalvez and M. de la Guardia, Elsevier (accepted for publication, October 2012).

Participation in international conferences and papers published in the proceedings of conferences

1. I. M. Apetrei, C.V. Popa (Ungureanu), D. Tutunaru, C. Apetrei, *Biosensors based on different carbonaceous materials for the analysis of biogenic amines*, **The Frontiers of Virtual Microscopy Conference**, Elsevier, March 21, 2012, Poster, http://www.materialstoday.com / virtualconference / Frontiers-of-the-microscopy
2. I. M. Apetrei, D. Tutunaru C.V. Popa (Ungureanu), C. Apetrei, *Electrochemical study of biogenic amines with conducting polymer sensors*, **International Conference of Applied Sciences, Chemistry and Chemical Engineering (CISA), Sixth Edition**, Bacau, April 24-27, 2012, Poster, http: // cisaconf.ub.ro

Article published: pp. 16-20, ISSN 2066-7817

3. I. M. Apetrei, D. Tutunaru, C.V. Popa (Ungureanu), C. Apetrei, *Development of amperometric biosensor based on immobilized tyrosinase in phosphate-doped polypyrrole film for detection of biogenic amines*, **14th International Meeting on Chemical Sensors - IMCS 2012**, May, 20-23, 2012, Nuremberg, Germany Poster, http://www.ama-science.org/home/details/1068

Article published: pp. 855-858, ISBN 978-3-9813484-1-5, DOI 10.5162 / IMCS2012 / P1.1.16

4. C.V. Popa (Ungureanu), I. M. Apetrei, D. Tutunaru, C. Apetrei, Biosensing properties of biogenic amines Towards novel Biosensors, **1st International Conference on Analytical Chemistry RO - ICAC'2012**, September 18-21, 2012, Targoviste, Romania, Poster, Best Poster Award, http: //www.icstm.ro/ICAC2012

5. I. M. Apetrei, D. Tutunaru C.V. Popa (Ungureanu), C. Apetrei, *Fish freshness Using chemical monitoring voltammetric electrodes modified*, **Centenary of Education in Chemical Engineering**, November 28 to 30, 2012, Iasi, Romania, Oral Presentation, http://www.ch.tuiasi.ro/ CNIC2012/index.html

6. C. Apetrei, *Biosensors based on nanotechnologies*, **Materials Today Virtual Conference: Nanotechnology**, Elsevier, December 11-13, 2012, Poster, http://www.materialstoday.com/virtual conference / materials-today-virtual-conference nanotechnology

Participation in national conferences

1. D. Tutunaru, I. M. Apetrei, *Applications of biosensors in medicine*, **Zilele Medicale Galatene**, November 6-7, 2012, Galati, Romania, oral presentation.

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