
Micro-Technological Steps During the Fabrication of an AcHE Biosensor Designated to the Environment Monitoring

Cristian Ravariu^{1,*}, Elena Manea², Alina Popescu², Cecilia Podaru², Catalin Parvulescu²

¹Dept. of Electronics, Polytechnic University of Bucharest, BioNEC Group, Bucharest, Romania

²Institute of Microtechnology, Bucharest, Romania

Email address:

cristian.ravariu@upb.ro (C. Ravariu), elena.manea@imt.ro (E. Manea)

To cite this article:

Cristian Ravariu, Elena Manea, Alina Popescu, Cecilia Podaru, Catalin Parvulescu. Micro-Technological Steps During the Fabrication of an AcHE Biosensor Designated to the Environment Monitoring. *American Journal of Bioscience and Bioengineering*. Special Issue:

Bio-electronics: Biosensors, Biomedical Signal Processing, and Organic Engineering. Vol. 3, No. 3-1, 2015, pp. 1-6.

doi: 10.11648/j.bio.s.2015030301.11

Abstract: Unfortunately, the pesticides pollute a large palette from environment: plants, organisms, soil and water. Using monitoring tools, like chemical biosensors, the use of pesticides can be put under control. Microelectronics offers the convenient transducers. Borrowing micro-technological processes, the enzymatic biosensors can be easily integrated onto the Silicon wafers. This paper proposes a biosensor for paraoxon pesticide detection, as a small piece from a decontamination soil technology plan. The paper reveals the pesticide detection principle, by paraoxon hydrolysis assisted by the Acetyl-cholinesterase enzyme, as key receptor. The enzyme is entrapped on a porous thin layer by adsorption. An advantageous method of the porous intermediate material anchored onto the Si-substrate, converting p-type Si in Si-porous by anodization, is described. Then, some technological steps, with tests and microscopy analysis are presented. Finally, the preliminary tests of the developed biosensor with the AcHE enzyme immobilized onto the Si-porous layer, are discussed.

Keywords: Biosensor, Design, Enzyme, Si-porous, Electrodes

1. Introduction

Nowadays agriculture cannot be conceived without the pesticides usage to protect the crops. The pesticide term includes chemicals substances intended for plant growth factor, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit, substances applied to crops either before or after harvest for preventing, destroying or controlling any pest. Organochlorines such as DDT were dominant in 1950, but they were replaced by organophosphates, carbamates after 1975, or new biopesticides, [1]. Unfortunately, the pesticides used in this field disturb not only the pest, but frequently cause the people health disturbing [2] and environmental damage, [3]. Using monitoring tools, like pesticides biosensors, the pesticides application can be set under control. Searching less toxic new generations of pesticides [4], the risks placed between consumers and environment may be reduced, especially on soil and water - the universal solvent - from the pesticides panel. Pesticides represent one of the main causes of the water pollution, reduce the nitrogen fixation and consequently

they reduce the biodiversity, destroy habitats especially for birds and threatens endangered species, [5].

Integrated biosensors usually contain onto the same chip the semiconductor solid-state support, the transducer as an electronic device and the biological detector as an enzyme [6, 7] or an antibody [8, 9]. This paper is starting from the possibility of pesticides hydrolysis, assisted by the Acetyl-cholinesterase enzyme as catalyst, [10]. Recently, an integrated capacitive electrode manufactured in the Si-technology, was proposed as simple and cheap biomedical transducer, [11]. On the other hand, some nanoporous materials compatible with the micro-technology process were used for the enzyme entrapping, [12]. Therefore, in this paper a more convenient porous material was investigated in respect with the Silicon technology. Finally, the biosensor with the AcHE enzyme immobilized onto a Si-porous layer and capacitive transducer that is sensitive to the pesticide concentration is discussed.

2. Key Elements of the Biosensor

2.1. AcHE Enzyme

Acetyl-cholinesterase, also noted AcHE, after the IUBMB Enzyme Nomenclature with the code - EC 3.1.1.7 - is an enzyme that degrades, through its hydrolytic activity, the acetylcholine neurotransmitter, producing choline and an acetate group. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity serves to the synaptic transmission, fig. 1.a. The AcHE has a very high catalytic activity — each molecule of AcHE degrades about 25000 molecules of acetylcholine per second, [13]. This action principle was extrapolated to the parasymphomimetic pesticides.

2.2. The Pesticide

The pesticides intensively used in the lasts twenty years are organophosphate, carbamates and organochlorines. They operate through the inhibition of acetyl-cholinesterase, allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms such as weakness or paralysis. They operate by disrupting the sodium/potassium balance of the nerve fiber, forcing to a continuously transmission through the nerve.

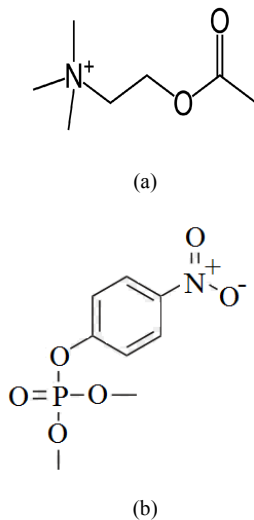


Figure 1. The molecule of (a) Acetylcholine; (b) Paraoxon.

Paraoxon is a novel generation of parasymphomimetic substance, which reacts as an acetyl-cholinesterase inhibitor, fig.1.b. Using a parasymphomimetic drug as a pesticide, the entire control of the parasymphomimetic nervous system (PSNS) can be transferred against pests, [14]. Chemicals from this group can act in two directions: (i) directly by stimulating the nicotinic or muscarinic receptors, or (ii) indirectly by the cholinesterase inhibition and supporting the acetylcholine releasing. An acetyl-cholinesterase inhibitor, abbreviated AcHEI or anti-cholinesterase is a substance that inhibits the cholinesterase enzyme action, preventing the acetylcholine breaking and favoring the degree and time action of the acetylcholine neurotransmitter.

Paraoxon is an organophosphate oxon and the active metabolite of the parathion pesticide. Paraoxon is one of the most potent acetyl-cholinesterase inhibitor available as insecticide, with a similar effectiveness to sarin, [13]. In aqueous dilutions, it represents a high risk of poisoning for humans or animals, due to its simply absorption through skin or teguments, in contact with the contaminated water from environment. Paraoxon exposure can result in headaches, convulsions, poor vision, vomiting, abdominal pain, severe diarrhea, unconsciousness, tremor, dyspnea, and finally lung-edema as well as respiratory arrest, [15].

As pesticide, the parathion is solved in water and generally is applied by spraying. It is often applied to cotton, rice and fruit trees. The usual concentrations of ready-to-use solutions are 0.05 to 0.1%, [16]. The chemical is banned for use on many food crops. After raining, the pesticide is accumulated in water and soil. Degradation of parathion leads to more water soluble products. Hydrolysis, which deactivates the molecule, results in diethylphosphate and 4-nitrophenol.

Degradation proceeds differently under anaerobic conditions: the nitro group on parathion is reduced to the amine.

2.3. The Si-porous Intermediate Layer

This paper proposes a biosensor for paraoxon pesticide detection, as a small piece from the decontamination soil technology plan, [17-21]. Some intermediate porous materials are frequently used in the biosensor construction, for the enzymes entrapping, [22]. Among these materials still exists: TiO₂ [23], Al₂O₃ [6], C-nanotube [24] or Si-porous [25]. The porous materials integration on a silicon wafer is starting by a first metal deposition, followed by subsequent processing steps, in order to convert them into compounds and finally into a porous matrix.

These intermediate porous materials augment the capillary, allowing the biomaterials entrapping in a liquid phase, during the pre-deposition technological stage. In the same time, the intermediate layer must be grown onto the Si wafer in order to be strongly anchored in substrate, to avoid accidental detachments during the applications.

Si-porous can be easily converted from a Si thin upper layer. Having a closer lattice constant with Si, we consider that the Si-porous stands for an intermediate material more convenient and more compatible with Si, from chemical and mechanical anchoring point of view.

The main advantages of the Si-porous intermediate material usage, between the Si-wafer and enzyme receptor, can be considered as:

- the Si-porous material preparation by anodization is a perfect compatible method with the microelectronics technology;
- the pore sizes can be simply adapted in respect with the anodization reaction parameters, changing the electrolyte composition;
- due to an increased area offered by the Si-porous material, against the Si-mono-crystalline, an enhanced miniaturization with capacitive electrodes can be

performed;

- in the Silicon micro-pores, some bio-sensitive materials can be entrapped, offering an enhanced mechanical stability and better attachment to substrate.
- the conversion of the Si-film into a Si-porous layer avoids further metal depositions and decreases the costs.

Therefore, the Si-porous was selected as intermediate layer for the AcHE enzyme entrapping. The solution is also in agreement with the nowadays tendency to integrate the biological receptors on the same chip with the microelectronics compounds that involves the Si-technology compatibility.

3. Key Technological Steps

3.1. Si-porous Conversion

As other authors claim, the Si-porous is a better alternative for the biological elements integration onto the Si-wafer. The receptor manufacturing is starting from the Si-porous preparation that must be able to capture the subsequent AcHE enzymatic layer.

The start wafers are 3 inch, Si-n type, <100>, 1-7 Ωcm. A thermal oxide is grown and patterned before the selective ion implantation. Then a high dose boron implantation on the front-side of the wafer occurs, in order to convert a thin upper layer of Silicon from n-type into p-type. In order to convert Si p-type in porous silicon, the electrolyte HF:CH₃COOH:H₂O with 180:60:60 ratios, at a current density > 1 mA/cm² is used. In this way, a porous silicon layer with a porosity of 56% can be accomplished.

The technological step of the Si-porous layer growing onto the Si surface consists in a thin Si layer conversion by anodic oxidation using an electrolyte 4% HF in DMF (Dimethylformamide), at room temperature and an optimum current intensity for the targeted pores uniformity and distribution. After the porosification process, some annealing processes help to the film stability: 450⁰C in H₂ followed by 950⁰C in N₂ environment.

The morphological characterization is developed by SEM techniques (fig. 2) and the porosity is gravimetric detected.

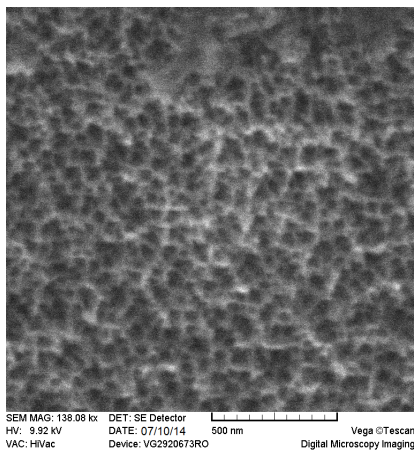


Figure 2. SEM images of the achieved Si-porous.

Table 1 presents the anodization results, in different conditions of duration (10min ... 120min) and reverse bias, for different current densities, in respect to the other author different conditions, [26].

Table 1. Si-porous processing parameters.

Current Density (mA/cm ²)	Center Pore Depth (µm)
2	34 ^[26]
6	12 ^[26]
10	2
50	8
100	40
300	50

The etching processing was accomplished under different current densities (10mA/cm²...300mA/cm²) and a time of 10min, assisted by illumination with a halogen lamp, Philips Dichroic, 50W, 12V. The experiments showed that the pore width increases from 2µm for (10mA/cm²... 20mA/cm²) and 3µm for (25mA/cm²...30mA/cm²). If the current density continue to increase more than 50mA/cm², many tiny pores are developed inside a main pore, rather with with a crater shape. The width of the main pore reaches 7µm for a current density about 300mA/cm². However, the width increasing in a next stage is accompanied by a decreasing of the thickness of the tube walls, probably due to the coalescence of the tiny pores. However, the current density of 50mA/cm² offered the optimum capilarity for the enzyme entrapping, [26].

3.2. Electrodes Design and Processing

The transducer of this biosensor is represented by an electrical capacitance. From the design stage, the capacitor has a constant armature surface and a fix distance between electrodes, so that any variation in capacitance reflects the electrical permittivity change of the material between electrodes. This permittivity variation is proportional with the quantity of ions accumulated after the enzymatic assisted reaction of the pesticide hydrolysis. Therefore, in order to increase the sensor sensitivity, as high as possible active area is demanded. In this sense, the electrodes are designed with an interdigitated geometry, fig. 3.

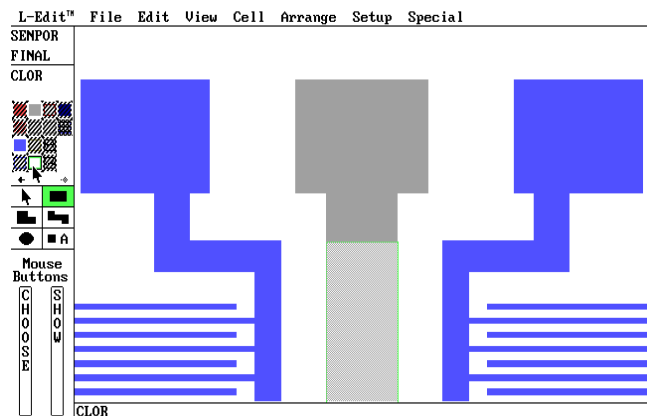
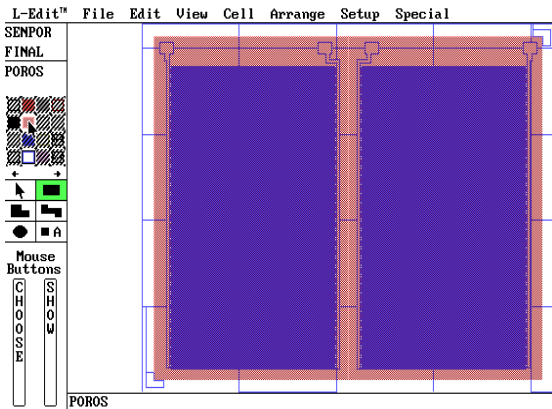


Figure 3. Electrodes design in L-Edit; the interdigitated geometry increases the active area and the sensitivity.

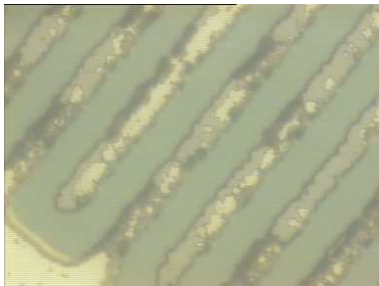
Figure 3 shows:

- (a) a detail of the Central METAL pad 144x144 μm , in the center region, grey color, as the reference electrode;
- (b) two METAL pads 144x144 μm , lateral positioned, in blue color, as separate cellule from the central electrode;
- (c) the interdigitated zones with METAL layout 8 μm width and 10 μm distance among them.

In figure 4a, the total metal area overcomes the total porous region, onto a chip of 160 μm and a total useful area of 4x4mm².



(a)



(b)

Figure 4. (a) Electrodes overlay the porous global active area; (b) detail of electrodes on the final product.

The final interdigitated structure comprises 98 horizontal metallic traces, which are starting from the central pillar for each electrode, detail in fig. 4b.

3.3. Enzyme Entrapping

The proposed biosensor is composed by a capacitive transducer integrated within the acetyl-cholinesterase enzyme as a receptor layer. The porous silicon represents an optimum intermediate material for the biosensor transducer, due to some advantages: (a) the preparation of Si-porous by anodic oxidation is completely compatible with the microelectronics technology and (b) the porous materials are perfect candidates for the enzyme immobilization by the adsorption method.

For the enzyme membrane preparation, the AcHE is immobilized in serum albumin from bovine provenience (BSA), using a glutaraldehyde solution (GA) - 2,5% concentration, as polymerization agent. The method allows the enzyme applying directly onto the Si/Si-porous surface. A

top view of the AcHE membrane is presented in fig. 5. All the time, a phosphate buffer solution ensures a constant pH=7.0.

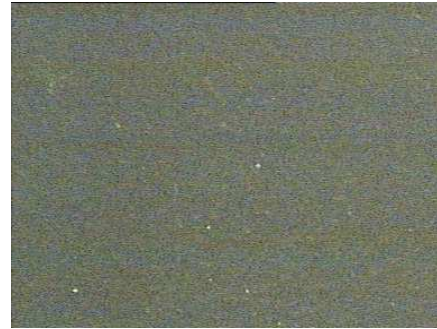


Figure 5. The enzymatic membrane view, after immobilization.

4. Discussions

After the enzyme entrapping, a preliminary C-V analysis was performed. Figure 6 presents the Capacitance-Voltage test curves of the biosensor in a primary stage, after the enzyme entrapping, without a pesticide solution.

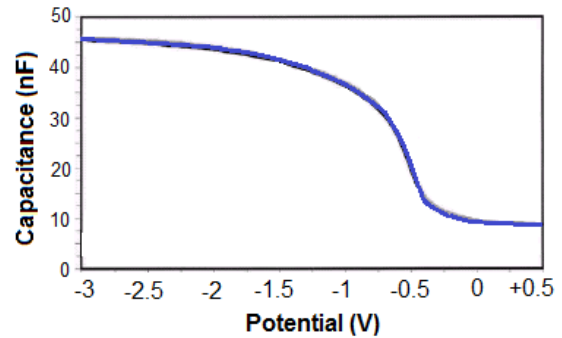


Figure 6. The C-V curves using the capacitive transducer and the enzyme membrane entrapped on Si-porous.

The maximum capacitance is about 45nF and the minimum is 7nF, as is predictable for a MOS structure. The shape of the C-V curve proves that our capacitive biosensor work as a generalized MOS capacitor, with enzyme on Si-porous on Si-substrate, instead of metal on oxide on silicon. The voltage ranging from negative values toward positive values bring the capacitor from the accumulation regime through the depletion regime (the middle decreasing part of the curves) toward the inversion regime. The C-V curve is almost reproducible that means an adequate enzyme entrapping onto the silicon surface.

5. Conclusions

This article discussed a biosensor of pesticides using AcHE enzyme on Si-Porous structure, as receptor element. This contribution presented the main technological steps and intermediate results. The paraoxone analyte and the Acetyl-cholinesterase key enzyme were revealed in their inter-dependency. The enzyme entrapping onto a Silicon-wafer was possible in good condition by an

intermediate Si-porous layer. The growing technological process of the Si-porous layer onto the Si surface is started by the conversion of the n-type wafer into a p-type thin layer at surface. The next process was the anodization. A short analysis concerned different electrochemical conditions: duration between 10min ... 120min, reverse biases and different current densities. The centre pore depth increased from 2 μ m for a current density of 10mA/cm² to 8 μ m for 50mA/cm² and reaches 50 μ m for 300mA/cm². However, the AcHE enzyme was optimum entrapped on Si-porous made at a current density of 50mA/cm², being in agreement with the literature.

The enzyme membrane immobilization technique was by adsorption onto the Si-porous layer and fixed with glutaraldehyde by the crosslink method.

Finally, the preparation of the capacitive electrodes as an interdigitated structure comprising 98 horizontal metallic traces was used to optimum increase the active area.

The C-V primary curve proves the sensor functionality as a generalized MOS structure. This preliminary test could represent for the next future, a starting point for a portable system development, simply to be used in any environmental applications.

Acknowledgements

The work has been supported initial by PNII Projects 12095, 62063, POSDRU/89/1.5/S/62557 and co-funded by PN2 contract no. 35 / 2014 EL081401 MEDICY.

References

- [1] Rao GVR, Rupela OP, Rao VR, Reddy YVR. Role of biopesticide in crop protection: present status and future prospects. *Indian Journal of Plant Protection*, 35 (1), 2007, pp. 1-9.
- [2] Jurewicz J, Hanke W. "Prenatal and childhood exposure to pesticides and neurobehavioral development: review of epidemiological studies". *Int J Occup Med Environ Health* 21 (2), 2008, pp 21-32.
- [3] Dudman NP, Jersey J, Zerner B. Organophosphate inhibitors. The stereospecificity of hydrolysis of methyl n-butyl p-nitrophenyl phosphate by serum phosphotriesterases (EC 3.1.1.2) and by acetylcholinesterases (EC 3.1.1.7). *Biochim Biophys Acta*. 1977, Mar 15, 481(1), pp. 127-139.
- [4] Gader Ghaffari, Farhad Baghbani, The Study of Important Agronomic Traits by Multivariate Analysis in Winter Rapeseed Cultivars, *American Journal of Bioscience and Bioengineering*. Science PG, Vol. 2, No. 1, 2014, pp. 15-17.
- [5] Palmer WE, Bromley PT, and Brandenburg RL. *Wildlife & pesticides - Peanuts*. North Carolina Cooperative Extension Service. Retrieved on 2007, pp. 10-11.
- [6] C. Ravariu, A. Popescu, C. Podaru, E. Manea and F. Babarada. The Nanoporous Al₂O₃ Material Used for the Enzyme Entrapping in a Glucose Biosensor. MEDICON Conference, XII Mediterranean Conference on Medical and Biological Engineering and Computing Proceedings, Chalkidiki, Greece, vol. 29, May 2010, pp. 459-462.
- [7] David A. Areshidze, Lyudmila D. Timchenko, Maria A. Kozlova, Protective Effect of Enzymatic Hydrolyzate of Chlorophytum Comosum (L.) against Experimental Toxic Liver Injury in Wistar Rats at the Age of Two Years, *American Journal of Bioscience and Bioengineering*. Science PG, Vol. 1, No. 4, 2013, pp. 49-54.
- [8] C. Ravariu, F. Ravariu. Some optimization of Bio-FETs with electrical charged bioliquid, *Romanian Reports in Physics*, Vol.58, No.2, 2006, pp.189-194.
- [9] Samson Ayodeji Olofinsae, Bartholomew Okechukwu Ibeh, Habu Josiah Bitrus, Jatinder Ahluwalia, Immunopharmacological Activity of Calcitriol Pre-Treated RAW264.7 Cell Line Stimulated by Phorbol 12-Myristate13-Acetate (PMA) and Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) Agonist, *American Journal of Bioscience and Bioengineering*. Science PG, Vol. 2, No. 4, 2014, pp. 55-59.
- [10] Kavitha CV, Gaonkar SL, Narendra Sharath Chandra JN, Sadashiva CT, Rangappa KS. Synthesis and screening for acetylcholinesterase inhibitor activity of some novel 2-butyl-1,3-diaza-spiro[4,4]non-1-en-4-ones: derivatives of irbesartan key intermediate. *Bioorg Med Chem*. 2007 Dec 1;15(23), pp. 7391-7398.
- [11] C. Ravariu, F. Babarada, E. Manea, C. Parvulescu, I. Rusu, I. Enache, M. Cristea, J. Arhip. Manufacturing of Capacitive Electrodes on Si-substrate for Electrophysiological Applications, 37-th IEEE International Annual Conference of Semiconductors, 13-15 Oct, 2014, Sinaia, Romania, pp. 151-154.
- [12] C. Ravariu, E. Manea, C. Parvulescu, F. Babarada, A. Popescu. Titanium dioxide nanotubes on silicon wafer designated for GOX enzymes immobilization, *Digest Journal of Nanomaterials and Biostructures*, vol. 6, no 2, April - June 2011, pp. 703 - 707.
- [13] Dale Purves, George J. Augustine, David Fitzpatrick, William C. Hall, Anthony-Samuel LaMantia, James O. McNamara, and Leonard E. White. *Neuroscience*. 4th ed., Sinauer Associates, 2008, pp. 121-123.
- [14] Fee D.C., Gard D.R., Yang C. *Phosphorus Compounds*. Kirk-Othmer Encyclopedia of Chemical Technology. John Wiley & Sons: New York, 2005.
- [15] S. Kegley, B. Hill, S. Orme. Parathion - Identification, toxicity, use, water pollution potential, ecological toxicity and regulatory information". *Pesticide Action Network*, 2010.
- [16] K Husain, RA Ansari, L Ferder Pharmacological agents in the prophylaxis/treatment of organophosphorous pesticide intoxication. *Indian Journal of Experimental Biology*, 2010 July, 48(7), pp. 642-650.
- [17] Cristian Ravariu. SOI devices - new challenges for Nano-Electronics, Workshop on Circuits, Systems and Information Technology WCSIT, Invited presentation, Iasi, Romania, IDEO Bussines Center, 3-4 July 2014, pp.11.
- [18] A. Bondarciuc, C. Ravariu, V. Bondarciuc, G. Alecu, Non-polluting medical technology for environment and patient used in inflammatory diseases monitoring, *Environmental Engineering and Management Journal*, accepted paper in 2014, in press.

- [19] A. Voina, G. Alecu, C. Ravariu, C. Voina, Monitoring system of air quality, 17.55-18.10, 24 July 2014, pp. 63, International Conference - New Trends on Sensing- Monitoring- Telediagnosis for Life Sciences- NT-SMT-LS, organised by Transilvania University of Brasov, Romania, during July 24-26, 2014.
- [20] Jong-Hun Park, Duckshin Park, Method of Removing Fe Particulate Matters from Subway Environments, International Journal of Environmental Monitoring and Analysis. Science PG, Vol. 3, No. 1, 2015, pp. 1-6.
- [21] Col Islam-ul-Haque, Asim Saleem, Community Based Sewage Treatment through Hybrid Constructed Wetlands System for Improved Health & Hygiene and for Enhanced Agriculture Productivity / Livelihood Generation in Rural Water Scarce Environments-Pakistan, American Journal of Environmental Protection. Science PG, Vol. 4, No. 1, 2015, pp. 45-54.
- [22] Ying Zhuo, Ya-Qin Chai, Ruo Yuan, Li Mao, Ya-Li Yuan, Jing Han, Glucose oxidase and ferrocene labels immobilized at Au/TiO₂ nanocomposites, with high load amount and activity for sensitive immunoelectrochemical measurement of ProGRP biomarker, Biosensors and Bioelectronics 26, 2011, pp. 3838-3844.
- [23] Jingxia Qiu, Shanqing Zhang, Huijun Zhao, Recent applications of TiO₂ nanomaterials in chemical sensing in aqueous media, Sensors and Actuators B 160, 2011, pp. 875-890.
- [24] Pablo R. Dalmaso, María L. Pedano, Gustavo A. Rivas, Supramolecular architecture based on the self-assembling of multiwall carbon nanotubes dispersed in polyhistidine and glucose oxidase: Characterization and analytical applications for glucose biosensing, Biosensors and Bioelectronics 39, 2013, pp. 76-81.
- [25] Saakshi Dhanekar, Swati Jain, Porous silicon biosensor: Current status, Biosensors and Bioelectronics, 41, March, 2013, pp. 55-64.
- [26] Tiffany M. Hoover, Development of macroporous silicon for bio-chemical sensing applications, 23rd Annual Microelectronic Engineering Conference, May 2005, pp. 78-86.