Covalent Immobilization of Peroxidase onto Hybrid Membranes for the Construction of Optical Biosensor

Lyubov Yotova[1*], Ahmed Hassaan[1], Spaska Yaneva[2]

[1]*Department of Biotechnology*
University of Chemical Technology and Metallurgy
8 Kl. Okhridski Blvd, 1756 Sofia, Bulgaria
E-mails: lkyotova@yahoo.com, ahmed.hassaan@ymail.com

[2]*Department of Fundamentals of Chemical Technology*
University of Chemical Technology and Metallurgy
8 Kl. Okhridski Blvd, 1756 Sofia, Bulgaria
E-mail: sp_yaneva@uctm.edu

[*]*Corresponding author*

Received: December 16, 2014 **Accepted: June 18, 2015**

Published: June 30, 2015

Abstract: *The aim of this study is to covalently immobilize horse radish peroxidase (HRP) onto new hybrid membranes synthesized by the sol-gel method based on silica precursors, dendrimers and cellulose derivatives. This new system will be used for designing biosensor. For investigation of the properties of membranes, HRP was used as a modeling enzyme. Kinetic parameters, pH and temperature optimum were determined, and the structure of the membranes surface was examined. Results showed higher relative and residual activity of HRP immobilized onto membranes with cellulose acetate butyrate with high molecular weight CAB/H. This novel biosensor could offer a simple, cheap and rapid tool with enhanced sensing performance as well as having potentials to find application in medicine, pharmacy, food and process control and environmental monitoring.*

Keywords: *Enzymes, Sol-gel, Mycotoxins.*

Introduction

Over the last decade biosensors research especially for optical biosensors emerged as a successful alternative to the other traditionally used expensive and complicated analytical techniques, where biosensors exhibit high specificity, efficiency, direct applicability, reliability and cost effectiveness towards detecting many compounds and samples contaminated with toxic compounds [2, 4, 9].

Sol-gel method revealed many advantages for development of biosensors like being versatile, simple and reproducible, to successfully produce materials with excellent surface properties, porosity and transparency [21]. Previous research papers in the area of biosensors reported using horseradish peroxidase (HRP) for detection of different substrates [3, 8].

Enzyme immobilization including attachment or incorporation onto or within solid matrices, via different approaches from adsorption, covalent attachment, confinement or entrapment became very popular in designing potential biosensors [1, 12, 26]. This immobilization helps overcoming enzyme limitations by reducing the required enzyme amounts, prolonging its lifetime, while increasing the potential for enzyme reuse [1] and enzyme stability [13, 14, 26]. While the potential applications of biosensors allow detection of wide range of compounds in food [20], pharmaceutics [10], environment [11] and industry [2, 22].

Nanostructured materials and that on micro scale were used as supports for enzyme immobilization [13, 26]. Using solid membranes as matrices for covalent immobilization, helps improving recovery of immobilized enzymes, this could optimize operational cost while keeping the enzyme active and preserved. Covalent immobilization process onto solid membrane involves immobilization of the enzyme onto the prefabricated membrane support and using the resulting conjugates for the sensing process [16].

Materials and methods

Reagents

Peroxidase isolated from horseradish (E.C. 1.11.1.7) were purchased from Sigma-Aldrich; methyltriethoxysilane (MTES) and trimethoxysilane (TMOS) from Merck; cellulose acetate propionate with low molecular weight (CAP/L) and cellulose acetate butyrate with high molecular weight (CAB/H), ~15000 and ~25000, respectively, PAMAM dendrimers (Polyamidoamine) second generation from Sigma-Aldrich; hydrogen peroxide solution (35%) and chloroform from Merck. Other chemicals were of analytical reagent grade. Double-distilled and deionized water was used.

Synthesis of hybrid membranes by sol-gel method

Three groups of new hybrid membranes were prepared by sol-gel method with participation of silica precursors TMOS and MTES according to the general procedure as illustrated in Fig. 1. Cellulose acetate propionate with low (CAP/L) and cellulose acetate butyrate with high (CAB/H) molecular mass were used dissolved in chloroform as the system's organic component. As well as PAMAM dendrimers that was used as a source of active groups for covalent immobilization. The precursors were hydrolyzed in methanol for TMOS and ethanol for MTES [24].

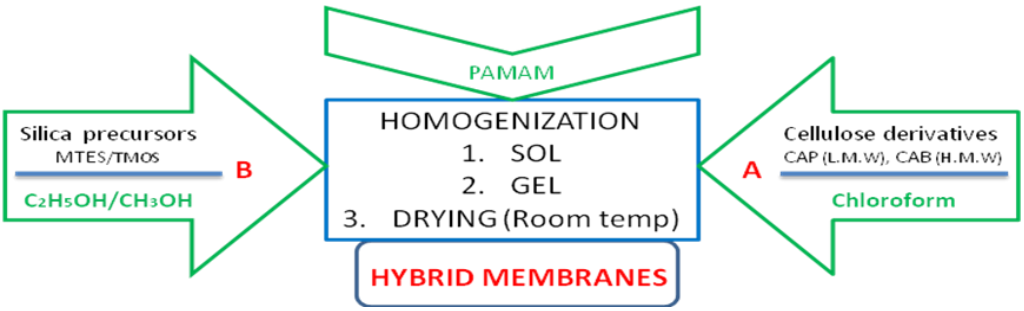

Fig. 1 Schematic illustration sol-gel synthesis of hybrid membranes

Group 1 (without silica precursors):

Chloroform was used as solvent for 1.5 g CAP/L or CAB/H, after the cellulose derivative being well dissolved, 50 µl PAMAM dispersed in chloroform was then added, and then stir mixture at room temperature for 3 hours. After stirring, solution poured, membranes formed and allowed to dry at room temperature.

Group 2 (With TMOS silica precursors):

Two solutions were prepared, A and B. Solution A was prepared as explained in Group 1, and solution B was prepared by dispersing 1 ml TMOS in 6 ml methanol while using concentrated HCl as a catalyst, and letting the resulting mixture to stir at room temperature for 90 minutes. After stirring, the two solutions were combined, stirred and dried as in Group 1.

Group 3 (With MTES silica precursors):
Group 3 was prepared like Group 2 but using MTES as the silica precursor.

Oxidation method for peroxidase
Oxidation of carbohydrate residues of peroxidase was done according to Zaborsky and Ogletree's method [21]. The oxidized enzyme was dialyzed using dialysis membrane from Serva, Germany, by submerging for 24 h in 50 mmol/L acetate buffer with pH 5.6.

Covalent immobilization of peroxidase onto hybrid membranes
The immobilization process to the support could be achieved through different approaches including Zaborsky and Ogletree method as well as glutaraldehyde method. Within Zaborsky and Ogletree method, enzyme immobilization onto activated support is achieved by covalent linkage between amino groups flanking onto the fabricated matrices to the carbohydrate residues existing within the enzyme structure [7]. The effect of various parameters, including pH, enzyme concentration and addition of functional additives was reported to have an effect on enzyme immobilization efficiency and catalytic properties.

Peroxidase immobilization was carried out in the following sequence: 1.0 g of the hybrid membranes was added to 10 ml of the oxidized dialyzed solution of peroxidase. Immobilization was done under continuous stirring for 24 h at 4 °C.

Determination of enzyme activity and content of protein
Peroxidase activity was determined spectrophotometrically at λ = 460 nm using H_2O_2 as a substrate. Total protein content was determined by modified Lowry [17] method using bovine serum albumin as a standard.

pH and temperature optimum
pH optimum and residual activity for free and immobilized HRP was determined at pH values from 4.0 to 8.0, and the temperature optimum was determined in the range from 20 °C to 60 °C.

Determination of Michaelis constant
Kinetic parameters for free and immobilized HRP were determined. The kinetic parameters of the different matrices used for immobilization were compared and evaluated against those parameters for the free HRP. Michaelis-Menten (K_m) constant of the enzymes was calculated using Matlab program.

Membranes imaging
Hybrid membranes structure was examined using microscope "Carlzeiss", Jena, Jenatech Inspection, monochromatic light source with CCD camera.

Results and discussion
The three groups of constructed hybrid membranes exhibited good uniformity, flexibility, with homogeneous distribution of the silica particles, transparency and surface properties. In addition, used cellulose derivatives and silica exhibit biocompatibility [15], in addition to their inertness towards the enzyme and being cheap. Using sol-gel process resulted in having hybrid membranes with good characteristics suitable for immobilization [7, 21].

It was reported that the used inorganic silica precursors is integrated within the cellulosic matrix forming hydrogen bonding connecting both organic and inorganic phases [23]. These uniformity and good surface characteristics was reported as one of the desirable criteria for sensing applications and enzyme immobilization [15]. Adding silica to membranes and sol-gel processing influenced the drying phase by overcoming the phenomena that some of the larger pores are emptied while smaller pores stay wet by the solvent that decrease the resulted internal pressure gradients. This resulted in better membrane properties and performance while enhanced drying process at room temperature within shorter time periods. Also using PAMAMs as being a polymeric material might affected the inorganic condensation-polymerization process [12].

Fig. 2 shows group (1) membranes, we can observe a high and uncontrolled porosity of the membranes with CAB/H compared to those with CAP/L that showed less and controlled porosity with good transparency. This coincides with previous work done on Ketotifen release from CAB and CAP membranes reporting higher release from CAB membranes versus uniform release from CAP membranes which is associated with the pores distribution [3]. On the other hand, no phase separation was observed between PAMAM and cellulose derivatives.

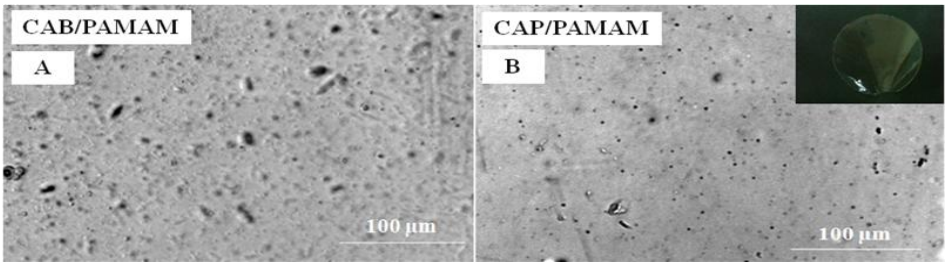

Fig. 2 Optical microscopy images for membrane surface. Magnification ×10, light field.

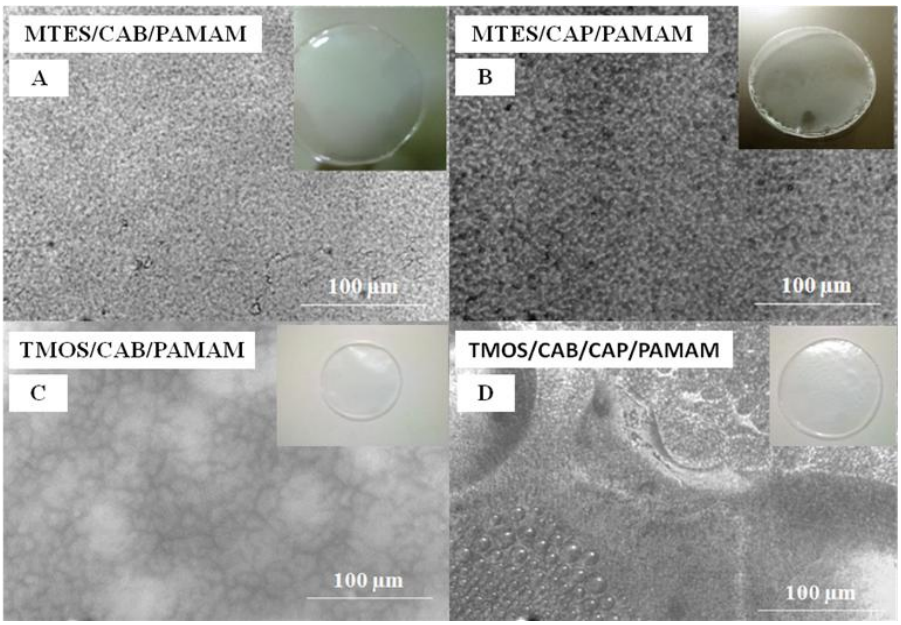

Fig. 3 Optical microscopy images for membrane surface. Magnification ×10, light field.

As shown in Fig. 3, TMOS/CAB/PAMAM synthesized membrane showed a uniformly and homogeneously pleated structure due to the used CAB/H, with no observed silica aggregates which resulted in a smooth like transparent structure. While both MTES/CAB/PAMAM and

MTES/CAP/PAMAM were similar showing homogeneous membrane structure, with well and homogeneous distribution of SiO_2 particles in a resulted grainy structure. On the other hand, synthesizing hybrid membranes using CAB and CAP in ratio 1:1, along with MTES precursors, showed phases separation of the two derivatives, resulting in non-homogeneous membrane structure, distinguishing CAP small uniform and CAB large uncontrolled porous structure.

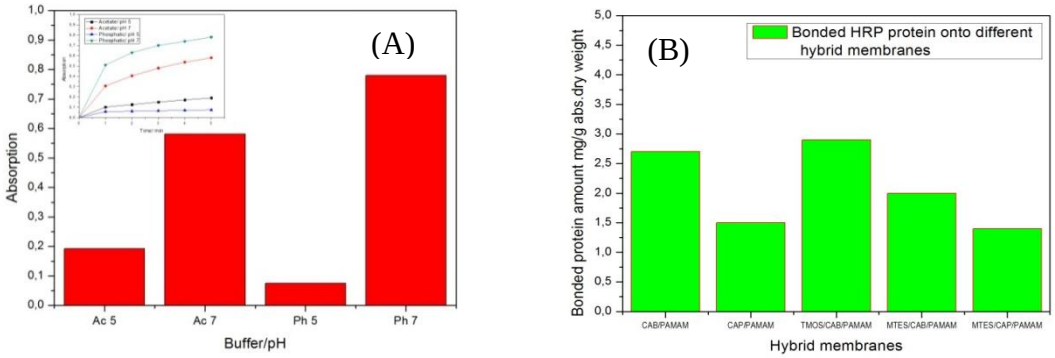

Fig. 4 (A) Immobilization efficiency and activity of HRP against the medium type and pH, (B) Bonded protein amount onto hybrid membranes at pH 7.0.

Fig. 4 (A) shows optimization step by evaluating resulted immobilization efficiency by optimizing the pH value and type of used buffer for the immobilization process. Comparison involved phosphatic and acetate buffers with different pH values, where phosphatic buffer with pH 7.0 resulted in the best immobilization and activity followed by acetate buffer with pH 7.0, while low immobilization efficiency was observed using lower pH. These results coincides with previous reported data where using pH near isoelectric point (ISP) of the used protein – that is around 7.0 in our case – results in better covalent immobilization on solid supports, while at other higher or lower pH values the immobilization yield dropped drastically [19]. This is principally because maximum adsorption of a protein can be achieved when it has a neutral charge, i.e. at the ISP. At other pH, the protein molecules will carry a charge and so repel from each other.

Conditions utilized for oxidation of carbohydrate residues of peroxidase were selected in a manner to maximally preserve enzyme activity, while obtaining sufficient reactive groups for immobilization. As reported previously, by using higher concentration of oxidizing reagent, a risk of activity loss exists [21]. Covalent binding of the enzyme to PAMAM dendrimers was established between the amide groups of PAMAM and the oxidized carbohydrate residues of HRP. This method was reported and applied in previous work, exhibiting an advantage that the immobilization does not change the conformation of the enzyme molecule nor interfering with active site as binding always takes place away from these active centers [15].

As shown in Fig. 4 (B), highest levels of HRP were found to be linked to the membranes containing TMOS/CAB/PAMAM, then CAB/PAMAM, followed by MTES/CAB/PAMAM. Results obtained for HRP specific and relative activity with membranes containing TMOS/CAB/PAMAM, and CAB/PAMAM were shown to be the highest as well. This due to the CAB higher available surface with its higher surface irregularities and porosity compared with CAP based membranes, which might be due to the different size of the butyrate group compared with the propionate group, having CAB membranes with more open structure at the end [3].

Immobilization of HRP on membranes obtained by the sol-gel method

Catalytic properties of free and immobilized HRP

Tables 1 and 2 present the HRP catalytic properties onto different membranes. Upon comparison, TMOS/CAB membranes showed better results, where the highest relative activity was found to be 58% and 62% for TMOS/CAB and CAB respectively, followed by CAP and MTES/CAB that recorded 54% and 53% respectively. The reason for this might be that CAB/H has high porosity with absence of aggregates as being elaborated previously, this enhanced substrate penetration into the membrane and the products out by decreasing diffusion limitation. While as reported before by Lagoa et al. [15] and Bayramoglu et al. [5], the inclusion of CAP/L in the hybrid membranes results in having more homogeneous structure with controlled porosity, this way it is believed that CAP/L exhibited diffusion limitations which also corresponds to results reported by Bayramoglu et al. [5].

Table 1. Catalytic properties of free and immobilized peroxidase onto different matrices

Sample	Abs drywt, [mg/g]	Specific activity, [U/mg]	Relative activity, [%]	pH optimum	Temp. optimum, [°C]
Free HRP	-	67	-	6.0	25
CAB/PAMAM	2.6	41.9	62	6.5	35
CAP/PAMAM	1.4	36.9	54	6.0	40
TMOS/CAB/PAMAM	2.7	39.2	58	6.0	40
MTES/CAB/PAMAM	1.9	35.7	53	6.0	40
MTES/CAP/PAMAM	1.3	19.2	28	6.0	40

In Table 2, kinetic parameters of the HRP immobilized on different membranes were compared with the parameters of the free HRP. TMOS/CAB/PAMAM and CAB/PAMAM showed the highest results compared to the free HRP, followed by the other membranes. Using Lineweaver Burk plotting for characterization of our immobilized HRP, both immobilized and free HRP exhibited response to the reduction of H_2O_2 with a recorded linear range from 3.4 µmol/l till 10.2 mmol/l. K_m values obtained were found varying between 6.3×10^{-3} and 28.7×10^{-3}. These obtained K_m values coincides with those obtained by Mohamed et al. [18] that were 5.5 and 3.6 mM for free and immobilized peroxidase respectively upon immobilization onto chitosan. These results show that our immobilized HRP might has a greater affinity to the substrate compared to the free HRP enzyme [12, 24].

Table 2. Kinetic parameters for free and immobilized peroxidase onto hybrid membranes

Sample	K_m, [µM]	V_{max}, [µM/min]	R^2
CAB/PAMAM	6.33	6.25	0.891
CAP/PAMAM	10.23	2.99	0.828
TMOS/CAB/PAMAM	6.9	6.88	0.975
MTES/CAB/PAMAM	12.14	5.72	0.981
MTES/CAP/PAMAM	28.7	5.43	0.977
Free HRP	19.1	42.11	0.945

It was observed that using cellulose derivatives and silica precursors in the gel formation process affected resulted pores dimensions and the matrix structure, which enhanced immobilized enzyme activity by facilitating substrate molecules diffusion through the membranes while receiving greater protection from the environment. This explains the

resulted increase in enzymatic activity in CAB/PAMAM, TMOS/CAB/PAMAM and MTES/CAB/PAMAM [6].

As shown in Fig. 5 and as a function of pH, 100% residual activity remained the same for all samples at optimum pH 6.0 except a shift to pH = 6.5 that was recorded by CAB/PAMAM. Immobilized HRP curves showed optimum pH range were shown to be wide, with relatively high residual activity over a wider pH range, which coincides with previous results [25].

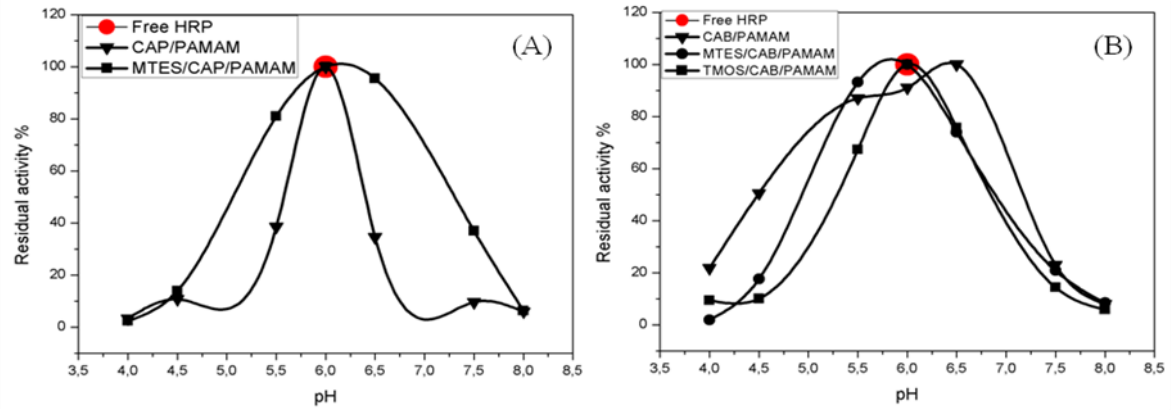

Fig. 5 pH effect on residual activity of immobilized HRP, compared to free HRP residual activity at optimum pH. Assay was performed at room temperature.

As shown in Fig. 6 and as a function of temperature, results showed a more general stability of HRP behavior in all systems by shifting of the temperature optimum from 25 °C for free HRP to 35 °C for CAB/PAMAM, and 40 °C for the other membranes, where matrix found to be influencing temperature optimums and enzyme stability, this observations corresponds with previous work results done by our group [26].

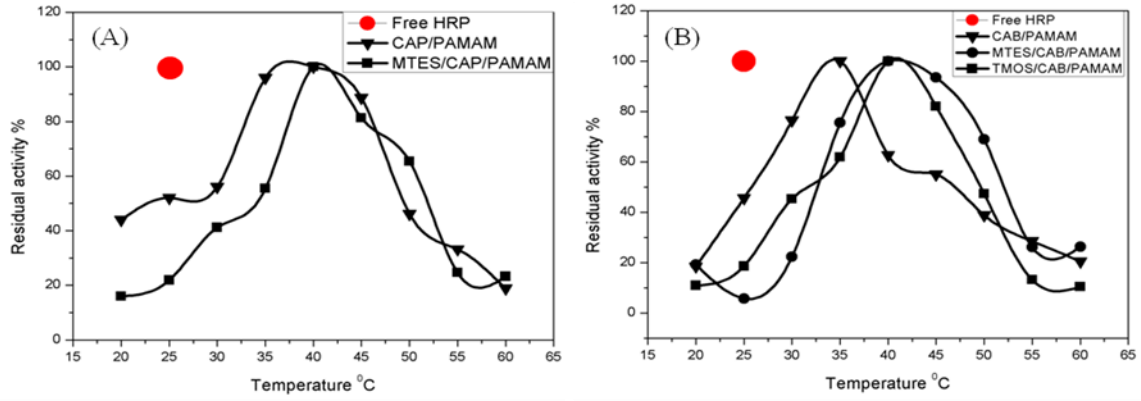

Fig. 6 Temperature effect on residual activity of Immobilized HRP, compared to free HRP at optimum temperature. Assay was performed at pH 6.0.

Conclusions

For obtaining system for efficient enzyme immobilization, membranes were prepared with participation of different silica precursors and HRP covalent immobilization was performed as a model enzyme. Analysis for immobilized enzyme onto different membranes showed change in pH and temperature optimums compared to those of free enzyme. The results showed that the highest relative activity were 58% and 62% for TMOS/CAB and CAB respectively. A better immobilization on matrices with CAB was observed. Efficiency of immobilization appeared to be depending on the type of used cellulose derivative, and conditions including

temperature and pH. The achieved system based on the optimized co-immobilized HRP demonstrated enhanced operational potential towards designing biosensor and further construction of immunosensor.

Abbreviations

CAB/H	–	cellulose acetate butyrate with high molecular weight
CAP/L	–	cellulose acetate propionate with low molecular weight
PAMAM dendrimer	–	poly (amidoamine) dendrimer
TMOS	–	trimethoxysilane
HRP	–	horseradish peroxidase
MTES	–	methylthriethoxysilane

Acknowledgements

The present work is supported by Project No. 11254, Scientific Research Sector (UCTM).

References

1. Abdullah J., M. Ahmad, Y. L. Heng, N. Karuppiah, H. Sidek (2006). Chitosan-based Tyrosinase Optical Phenol Bio-sensor Employing Hybrid Naflon/Sol-gel Silicate for MBTH Immobilization, Talanta, 70, 527-532.
2. Ajeet K., K. Sunil, V. Abhay, B. Shekhar (2013). Recent Advances in Detection of Ochratoxin-A, Open Journal of Applied Biosensor, 2, 1-11.
3. Arzum E., P. Aysun, M. Burcu, K. Kagan, Ö. Mehmet (2000). Electrochemical Biosensor Based on Horseradish Peroxidase for the Determination of Oxidizable Drugs, Turk J Med Sc, 349-354.
4. Banuls J. M., R. Puchades, Á. Maquieira (2013). Chemical Surface Modifications for the Development of Silicon-based Label-free Integrated Optical (IO) Biosensors: A Review, Analitica Chimica Acta, 777, 1-16.
5. Bayramoglu G., A. Akbulut, Y. M. Arica (2013). Immobilization of Tyrosinase on Modified Diatom Biosilica: Enzymatic Removal of Phenolic Compounds from Aqueous Solution, Journal of Hazardous Materials, 245, 528-536.
6. Brena B. M., B. V. Francisco (2006). Immobilization of Enzymes and Cells, Methods in Biotechnology, 15-26.
7. Brodelius P., K. Mosbach (1987). Immobilization Techniques for Cells/Organelles, Methods in Enzymology, Vol. 135, (Mosbach K., Ed.), Academic Press, London, 173-454.
8. Buddhalee P., M. Amatatongchai (2013). A Novel Hydrogen Peroxide Biosensor Based on Horseradish Peroxidase Immobilized on Poly (aniline-co-o-aminobenzoic acid) Modified Glassy Carbon Electrode Coated with Chitosan Film, Int J Electrochem Sci, 8, 10250-10264.
9. Coulet P. R. (1991). What is a Biosensor? Biosensor Principles and Applications, Marcel Dekker Inc., New York, Basel, Hong Kong, 1-6.
10. Deshpande M. V., E. A. H. Hall (1990). An Electrochemically Grown Polymer as an Immobilization Matrix for Whole Cells: Application in an Amperometric Dopamine Sensor, Biosens Bioelectron, 5, 431-448.
11. Erdem A., N. Altınığne, E. Kılınç, L. Gokgunnec, T. Dalbastı, M. Ozsoz (1998). Amperometric Biosensor Based on Mushroom Tissue Tyrosinase for the Determination of Phenolic Compounds, J Pharmaceutical Society of Ankara, 23, 1-6.
12. Gupta R., N. K. Chaudhury (2007). Entrapment of Biomolecules in Sol-gel Matrix for Applications in Biosensors: Problems and Future Prospects, Biosensors and Bioelectronics, 2387-2399.

13. Jiang Y., C. Guo, H. Xia, I. Mahmood, C. Liu, H. Liu (2009). Magnetic Nanoparticles Supported Ionic Liquids for Lipase Immobilization: Enzyme Activity in Catalyzing Esterification, Journal of Molecular Catalysis B: Enzymatic, 58(1-4), 103-109.
14. Jungbae K., W. G. Jay, W. Ping (2006). Nanostructures for Enzyme Stabilization, Chemical Engineering Science, 61, 1017-1026.
15. Lagoa R., D. Murtinho, M. H. Gil (1998). Membranes of Cellulose Derivatives as Supports for Immobilization of Enzymes, American Chemical Society, 299-308.
16. Liu X., J. Xing, Y. Guan, G. Shan, H. Liu (2004). Synthesis of Amino-silane Modified Superparamagnetic Silica Supports and Their Use for Protein Immobilization, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 238(1-3), 127-131.
17. Lowry O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall (1951). Protein Measurement with the Folin Phenol Reagent, J Biol Chem, 193, 265-275.
18. Mohamed S. A., S. A. Aly, T. M. Mohamed, H. A. Salah (2008). Immobilization of Horseradish Peroxidase on Nonwoven Polyester Fabric Coated with Chitosan, Appl Biochem Biotechnol, 144, 169-179.
19. Monk D. J., D. R. Walt (2004). Optical Fiber-based Biosensors, Anal Bioanal Chem, 379(7-8), 931-945.
20. Ozsoz M., J. Wang (1991). Tomato-seed-based Amperometric Sensor for the Determination of Alcohols, Electroanalysis, 3, 655-658.
21. Trevan M. (1980). Techniques of Immobilization, In: Immobilized Enzymes. An Introduction and Applications in Biotechnology, Wiley, Chichester-New York, 1-9.
22. Wang J., M. S. Lin (1989). Horseradish-root-modified Carbon Paste Bioelectrode, Electroanalysis, 1, 43-48, doi: 10.1002/elan.1140010107.
23. Xie K., Y. Yu, Y. Shi (2009). Synthesis and Characterization of Cellulose/silica Hybrid Materials with Chemical Crosslinking, Carbohydrate Polymers, 78(4), 799-805.
24. Yaneva S., D. Marinkova, L. Yotova, B. Samuneva (2007). Immobilization of Biocatalysts and Cells on Hybrid Membranes Syntheses on Sol-gel Method, Int J Bioautomation, 8, 172-183.
25. Yin H., S. Ai, W. Sha, L. Zhu (2009). A Novel Hydrogen Peroxide Biosensor Based on Horseradish Peroxidase Immobilized on Gold Nanoparticles-silk Fibroin Modified Glassy Carbon Electrode and Direct Electrochemistry of Horseradish Peroxidase, Sensors and Actuators B: Chemical, 137(2), 747-753.
26. Yotova L., S. Yaneva, D. Marinkova, S. Serfaty (2013). Co-immobilization of Peroxidase and Tyrosinase onto Hybrid Membranes Obtained by the Sol-gel Method for the Construction of an Optical Biosensor, Biotechnology & Biotechnological Equipment, 27(3), 3885-3889.

Prof. Lyubov Yotova, Ph.D.
E-mail: lkyotova@yahoo.com

Professor Dr. Lyubov Yotova, Department of Biotechnology, E.C.T.S. Expert of the Faculty of Chemical and System Engineering of University of Chemical Technology and Metallurgy, Sofia. *Professional Teaching Experience Areas*: Biotransformation Analyses of Bioproducts, Biosensors, including guest-professor at the European Biotechnology Master Course at the University of Perugia, Italy. *Scientific and research interests*: Biotransformations with immobilized biocatalysts for industrial and environmental application; biosensors for medicine, foods and environmental pollutants. Total number of publications – 82, 1 textbook; total number of research projects – 18, at the moment supervisor of 3 national projects with international participation; coordinator of NATO Project with the University of Manchester (2000-2003); coordinator of 3 projects Erasmus Networks in Biotechnology; coordinator of 6th FP Project Marie-Curie Action; work package leader of "Biosensor application in medicine, food and environment", project № 134310-LLP-2007-1-IT Erasmus-ENW – Eurobiotech (2007-2010).

Ahmed Hassaan, Ph.D. Student
E-mail: ahmed.hassaan@ymail.com

Ahmed Hassaan, Ph.D. student, Biotechnology Department, University of Chemical Technology and Metallurgy, Sofia. Received B.Sc. degree of Biotechnology in 2009, finished M.Sc. degree of Nanotechnology by 2013. Since 2007 he has worked at different scientific projects. Registered for a patent in cancer treatment, obtained Nanotechnology Non-degree Certification from Ludwig Maximilian Universitat (LMU), Munich, Germany. Participated in research work at LMU, Munich in the field of nano-drug delivery, and in advanced practice in nuclear medicine and proton therapy to cure cancer at Joint Institute of Nuclear Research, Russia. His scientific interests are in the field of Biosensors, immunotherapy, and Development of nanosystems.

Assist. Prof. Spaska Yaneva, Ph.D.
E-mail: sp_yaneva@uctm.edu

Spaska Yaneva is an Assistant Professor in Department "Fundamentals of Chemical Technology" in University of Chemical Technology and Metallurgy (UCTM), Bulgaria. She received B.Sc. degree, specialty Organic Synthesis, and M.Sc. degree, specialty Biotechnology, in the same university. Spaska Yaneva defended Ph.D. thesis in UCTM under the supervision of Prof. Yotova. Her scientific interests are in the field of immobilized enzymes, biosensors, environmental monitoring, toxic compounds and safe labor conditions.