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Silica-based hybrid materials as biocompatible coatings for xenobiotics sensors

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In the recent years many types of biosensors have been developed and used in a wide variety of analytical settings with applications in biomedicine, health care, drug design, environmental monitoring, and detection of biological, chemical and toxic agents.

Tailored surface properties such as tunable reactivity, biocompatibility or wettability could be obtained by different approaches of surface modification, so that the design of biofunctional surface is of great interest in bioanalysis research.

A good combination of support material and immobilization methods is of fundamental importance to achieve the desired performances from the sensing system.

The aim of this research is immobilization of tyrosinase onto silica hybrid membranes based on ethyltrimethoxy silane (ETMS) and methylthrietoxy silane MTES and cellulose derivatives. Tyrosinase was covalently immobilized by acrylamide/acrylonitrile copolymer included on hybrid membranes. pH and temperature optimum were determined for immobilized tyrosinase preparations as well as for the free enzyme.

Key words: hybrid membranes, enzymes, optical biosensors.

INTRODUCTION

Organic/inorganic hybrid materials prepared by the sol–gel approach have rapidly become a fascinating new field of research in materials science.

Organic molecules other than the solvent can be added to the sol and become physically entrapped in the cavities of the formed network upon gelation where the molecules have to endure the pH of the environment [1]. Most sol–gel bioencapsulates reported to date have used inorganic materials or carbon composite derived from either ethyltrimethoxy silane (ETMS) or methylthrietoxy silane (MTES) [2, 3]. The use of some organic molecules in the gel formation process that may influence the dimensions of the forming pores represents another way to increase the immobilized enzyme activity. Within such meso-or macroporous silica gels, substrate molecules diffusion is easier, explaining the increase of the enzymatic activity [4].

Porosity is a feature that allows analyte molecules to diffuse into the matrix and react with the enzymes or another biomolecules. As it was mentioned, many different molecules can be incorporated into the sol–gel matrix. Surface characteristics as well as uniformity in monoliths/thin films are one of the desirable criteria for sensing applications [5–6].

During the drying phase, some of the larger pores are emptied while smaller pores remained wet by the solvent, creating large internal pressure gradients. This stress causes cracks in large monoliths and is responsible for fractures in dry monolithic sensors upon immersion in water. Further addition of polymers as poly dimethil siloxane, polyamides, polyacrilates and polyethylene glycol (PEG) provide regulation of inorganic condensation-polymerization process and is also under investigation for improving sol-gel material. Polyethers were also used in sol-gel processing mixtures to control pore size distribution [7].

Phenols due to their toxicity, persistence and common occurrence in the biosphere are one of the most important groups of ecotoxins. These compounds are in a common use such as ingredients (components) and precursors of other chemicals including organic polymers, solvents, dyes (aminophenols), explosives (nitrophenols), surfactants (alkylphenols) or drugs [8].

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Photometric analyses by standard methods are commonly used for determination of phenols, and these analyses usually require sample pretreatment by filtration and distillation. Recently, tyrosinase based biosensors have been shown to be useful for this purpose. Easy fabrication, fast analysis, and low-cost are the main advantages of the biosensor method [9, 10].

The optical sensing techniques, in comparison with electrical methods, have some advantages, such as selectivity. They are also sensitive, inexpensive, non-destructive, and have wide capabilities. The idea behind these sensors is based on changes of optical parameters of sensing molecules entrapped usually in thin films. Optical fibers used in optical sensors to transmit the light in and out of the detection area assure such advantages as flexibility, directionality, low signal losses, low costs, etc. Since then, this technology is widely applied among others for immobilization of active molecules onto the tip of an optical fiber to fabricate a point sensor [11]. The most popular chemical immobilization utilized for the preparation of enzyme-based optical fiber sensors is the covalent coupling of enzymes to polymeric support [12].

This technique offers the most stable immobilized enzyme preparation by which the immobilization process is not easily reversed by pH, ionic strength, temperature, or solvent variations [13]. The main advantages of the biosensors over other kinds of sensors are their specificity of response and in some cases, their ability to work in very dirty environments [14].

Tyrosinase (EC 1.14.18.1, monophenol monooxygenase) is an enzyme which catalyzes the incorporation of molecular oxygen into phenolic compounds. Tyrosinase active site contains a coupled binuclear copper complex (type 3 copper). Recently, the mechanism of catalytic function of tyrosinase has been proposed and actively investigated. [15–17].

One of the key issues to develop biosensing platforms concerns the processes involved in enzyme immobilization on surfaces. The understanding of their fundamentals is crucial to obtain stable and catalytically active protein layers for developing successful biosensing devices [18, 19].

EXPERIMENTS AND EQUIPMENT

Reagents

Tyrosinase isolated from mushrooms, (E.C.1.14. 18.1) was supplied by Sigma-Aldrich; L-DOPA (L-3,4-dihydroxyphenylalanine) by Fluka; ethyltrimethoxy silane (ETMS) and methylthrietoxy silane (MTES) by Merck; cellulose acetate propionate with high and low molecule weight (CAP/H (~25 000 m.w.) (CAP/L (~15 000 m.w., respectively) by Sigma-Aldrich, copolymer from acrylamide and acrylonitrile (AA) were provided by the Biotechnology Department of UCTM, Sofia, Bulgaria [20], dimethyl formamide (DMF) by Merck.

Synthesis of hybrid membranes by the sol-gel method and visualization of the sufraces

Groups of hybrid materials were synthesised by the sol-gel method with the participation of silica precursors ETMS and MTES. Cellulose acetate propionate with high molecule weight CAP/H and CAP/L were used as an organic component of the system. A third component was included in the system as a carrier of active groups for covalent immobilization, namely copolymer of acrylamide/ acrylonitrile (AA). The precursors were hydrolysed in methyl alcohol for ETMS and ethyl alcohol for and MTES. Dimethylformamide was used as a solvent of the organic component. Hybrid membranes contained 5 ml ETMS (MTES), 3 g cellulose acetate propionate with high (low) molecular weight and 100 mg copolymer from poly-acrylamide and poly-acrylonitrile. The quantities and components for the synthesis and the conditions are described in a previous paper [21].

For visualization of membranes surfaces a microscope “Carlzeiss”, Jena, Jenatech Inspection, monochromatic light source “Infinity 22” with CCD camera – “Lumenera”, Canada was used.

Oxidation method for tyrosinase and covalent immobilization

Oxidation of the carbohydrate residues of tyrosinase was done with periodic acid according to Zaborsky and Ogletree’s method (0.04 mM in 0.05 mM acetate buffer, pH 5.0, in the dark) [28]. The oxidized enzyme was dialysed in a dialysis membrane from Serva, Germany by submerging in a 50 mM phosphate buffer with pH = 6.0 for 24 h. The immobilization of tyrosinase was carried out in the following sequence: 1.0 g of the hybrid membranes was added to 20 mL of the oxidized dialysed solution of tyrosinase. Immobilization was done under continuous stirring for 24 h at 4 °C.

Spectrophotometrically measurement of enzymatic activity and determination content of protein

Diphenolase activity was determined spectrophotometrically with 10 mM substrate L-DOPA as a substrate, at 25 °C, using spectrophotometer with

optical fibers (AvaSpec, Avantes, USA). The diphenolase activity does not show any lag period. The dopachrome assay was performed. The increase in absorption at 475 nm, due to the formation of dopachrome (ε 475 = 3 600 $M^{-1}cm^{-1}$), was monitored as a function of time. The activity is expressed as mole of L-DOPA oxidized per minute.

The total content of protein in the immobilized enzyme was determined by the Lowry [23] modified method using bovine serum albumin as a standard.

pH and temperature optimum

For determination of the pH optimum for tyrosinase, the residual activities of free and immobilized enzymes were determined in the sodium phosphate buffer with pH range from 5.0 to 8.0. To determine the temperature optimum for tyrosinase, the residual activities of free and immobilized enzyme were determined in the range from 20 °C to 50 °C.

RESULTS AND DISCUSSION

Groups of hybrid membranes were synthesised using different silica precursors. All membranes are mechanically resistant plastic and transparent, which is a necessary condition for the experiment. In such hybrid materials is possible to expect very interesting characteristics that are not found in organic polymer or inorganic material independently. For example, they can have features such as plastics flexibility and have excellent mechanical strength and thermal stability in the same time [24]. On the surface of the membranes different size aggregates (from 20 to 100 μm) are observed. For visualization of the surface series of images were made by microscope at different magnification. On Figure 1 images of the membranes surfaces are presented.

A comparison of the catalytic properties of immobilized enzymes on hybrid membranes containing ETMS and MTES CAP/L or CAP/H was made. In the table below catalytic properties of free tyrosinase and tyrosinase immobilized onto matrices are presented (Table 1).

The covalent binding of the enzyme to copolymer of acrylamide/acrylonitrile is effected between the amide groups of the copolymer and the oxidized carbohydrate residues of the enzyme. This method was applied in previous research as well. The advantage of the method is that immobilization does not change the conformation of the enzyme molecule and binding always takes place outside the active centres [20].

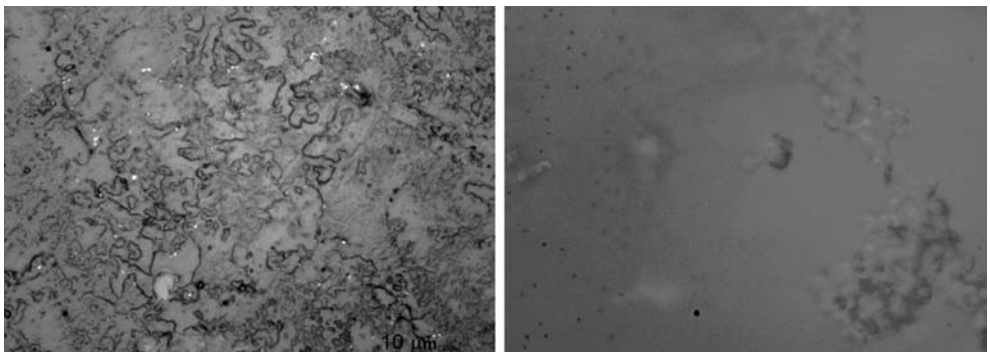

Fig. 1. Optical microscopy image of the surface on ETMS – left and MTES – right side at magnification ×50, light field

Table 1. Catalytic properties of free and immobilized tyrosinase

Membrane	Amount of bound protein [mg/g]	Specific activity [U/mg]	Relative activity [%]	pH optimum	Temperature optimum [°C]
Free tyrosinase	–	421	–	6.0	30
ETMS/CAP/H/AA	1.82	322	76.48	6.5	35
MTES/CAP/H/AA	2.34	312	74.10	5.5	35
ETMS/ CAP/L/AA	1.91	317	75.29	7.5	35
MTES/CAP/ L/AA	2.06	306	72.68	7.0	30

The results for specific activity of immobilized membranes are identical with results described in our previous work with substrate L-tyrosine [25]. For L-DOPA substrate tyrosinase immobilized onto hybrid membranes shows higher parameters of relative activity. Researchers have demonstrated that the silica sol-gel materials can retain the catalytic activities of enzymes to a large extent. The inorganic silica sol-gel material is biocompatible, has high thermal stability, chemical inertness and negligible swelling in non-aqueous solutions [26].

One of the most important parameters to be considered in enzyme immobilization is storage stability. The stabilities of the free and immobilized tyrosinase were determined after stored in phosphate buffer solution (50 mM, pH 6.5) at 4 °C for a predetermined period. Under the same storage conditions, the activities of the immobilized tyrosinase preparations decreased slower than that of the free tyrosinase. The free enzyme lost all of its activity within 4 weeks. The immobilized tyrosinase preserved its initial activity during several months storage period [27], which corresponds with the data reported by other authors [28]. On the figure below stored stability of immobilized tyrosinase activity are presented (Fig. 2).

The enzyme activities were seriously affected by the buffer solution pH value [29], so the effect of the pH value of buffer solution was investigated in the range from 5.0 to 8.0.

On the figure below pH profile of free tyrosinase and immobilized onto hybrid matrices are presented (Fig. 3).

The change in optimum pH depends on the charge of the enzyme and/or of the matrix. This change is useful in understanding the structure-function relationship of the enzyme and helps the activity of free and immobilized enzyme as a function of pH. As seen in Fig. 2, the optimum pH for free enzyme was found to be 6.5. In the case of MTES/CAP/H/AA immobilized tyrosinase the optimum pH shifted by 1.0 unit toward the acidic region. The results are the same that reported from *Yahsi and co-authors.* These shifts could be attributed to secondary interaction such as ionic and polar interactions, hydrogen bonding, etc. between the enzyme and the hybrid membrane [30].

On the figure below temperature profile of free tyrosinase and immobilized onto hybrid matrices are presented (Fig. 4).

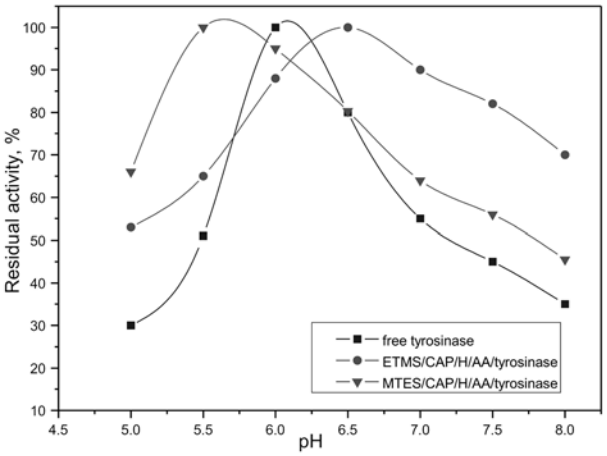

Fig. 3. Residual activity of free and immobilized tyrosinase as a function of pH

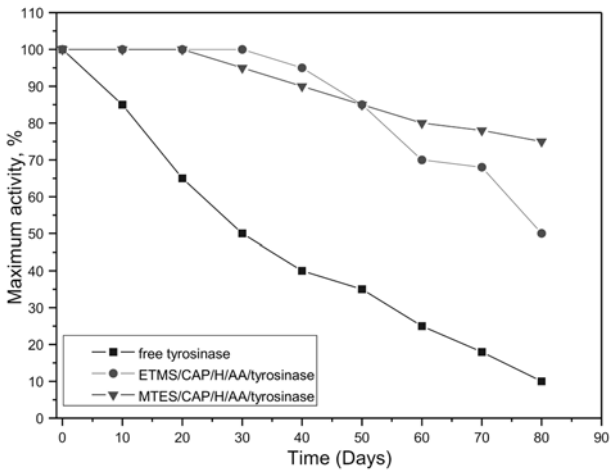

Fig. 2. Stored stability of immobilized tyrosinase activity

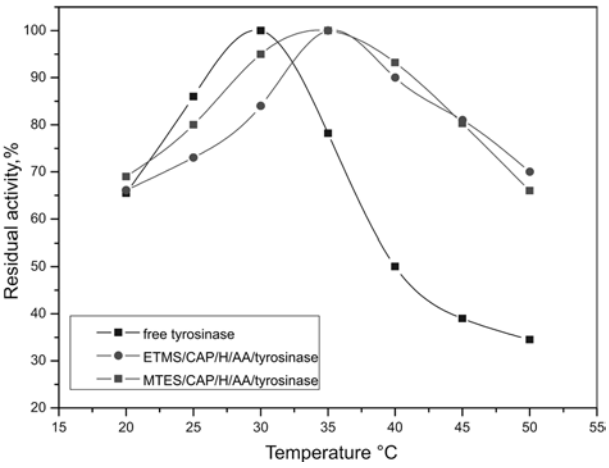

Fig. 4. Residual activity of free and immobilized tyrosinase as a function of temperature

Maximum enzymatic activity was obtained at 35 °C for immobilized enzymes. The optimal reaction temperature was higher than that of the commercial enzymes in its free form or when immobilized on other supports [31, 32], which illustrates a substantial degree of enzyme stabilization [33]. The stability of the obtained preparation demonstrates the advantages of immobilization onto hybrid membranes.

CONCLUSION

In order to construct an optical biosensor, membranes were synthesised with participation of different silica precursors and covalent immobilization was performed. The analysis of the enzymes immobilized on the different membranes showed change in pH optimums for ETMS/CAP/H/AA pH=6.5 and MTES/CAP/H/AA, pH=5.5 and the temperature optimums t = 35 °C for the same membranes compared to the characteristics of the free enzymes. The present research showed that the best membranes with the highest relative activity is (ETMS/CAP/H/AA – 76.48%) for L-DOPA substrate. The efficiency of immobilization depends on the type of the silica precursors and cellulose derivatives, when the other conditions are identical. The constructed optical biosensor based on covalent immobilized enzyme demonstrated excellent operational parameters.This membranes can be potentially applied for biosensors design for analyses in organic solvents, analysis of food and in monitoring of the environment.

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ХИБРИДНИ МАТЕРИАЛИ НА ОСНОВАТА НА СИЛИЦИЕВ ДИОКСИД КАТО БИОСЪВМЕСТИМИ ПОКРИТИЯ ПРИ БИОСЕНЗОРИ ЗА КСЕНОБИОТИЦИ

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(Резюме)

През последните години са разработени много видове биосензори с приложения в медицината, контрол на здравето, при създаването на нови лекарства, за екологичен мониторинг, за регистриране на биологични, химични и токсични агенти и други.

Адаптирането на повърхността на носителя, за да се подобри неговата реакционна способност, биосъвместимост и омокряемост, може да се постигне чрез различни методи за модифициране. Така се създава биофункционална повърхност, което е обект на изследователски интерес. Изборът на носител и метод за имобилизация са от основно значение за постигане на желаната ефективност на сензорната система.

Целта на това изследване е имобилизация на тирозиназа върху хибридни мембрани на основата на етилтриметокси силан (ETMS) и метилтриметокси силан (MTES) и целулозни производни. Тирозиназата бе ковалентно имобилизирана с помощта на съполимер на акриламид/акрилонитрил, включен в хибридния материал. Бяха определени pH и температурният оптимум на свободния и имобилизиран ензим.