Bioautomation, 2007, 8, Suppl. 1, 172 – 183 ISSN 1312 – 451X

Immobilization of Biocatalysts and Cells on Hybrid Membranes Syntheses on Sol-gel Method

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Summary: The investigations in the area of enzyme action in the living organisms give us the opportunity for applications of these biochemical catalysts in the different purposes of medicine, industry and analytical practice. Using of soluble enzymes is connected with many difficulties, because the enzymes are no regenerative and they are instability. By immobilization of the enzymes on to different carriers the more of these problems are overcome. The application of soluble enzymes caused many difficulties, by the reason that the enzymes can not be regenerated. The immobilization of the enzymes by means of different reagents and carriers overcomes this problem. The aim of this study to develop a sol-gel method of synthesis of new hybrid membrane, with immobilized biocatalysts (microbial cells and enzymes) for biosensor construction. This study shows that hybrid organic-inorganic membranes were synthesized.

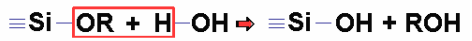
Keywords: Enzyme, Sensor, Sol-gel Processes, Biocatalysts.

1. INTRODUCTION

Organic-inorganic polymer hybrids are a new type of composite materials, in which the organic and inorganic components are combined at the molecular level [1]. There were published a lot of applications of these hybrid materials as functional coatings on glass and polymer substrates (bulks, powder and layers). In particular, these organic-inorganic polymer hybrids could potentially yield transparent, abrasion-resistant materials. They possess interesting properties such as molecular homogeneity, transparency, flexibility, and durability. Such materials could be employed in various applications, e.g. solid state lasers (optical components), replacements for silicon dioxide as an insulating material in the microelectronic industry, anti-corrosion coatings, scratch resistant coatings, contact lenses, host materials for chemical sensors, and membrane materials [2-7].

In these organic-inorganic polymer networks, formation of an inorganic network occurs through sol-gel processes [8]. The organic phase of the organic-inorganic polymer hybrid is synthesized "*in situ*" in liquid hydrolysed silica. The sol-gel method is widely used for preparation of hybrid materials, since it has the advantage of being a low-temperature process and potentially giving highly homogeneous nonmaterial. The chemical reactions involved in the sol-gel process are as follows:

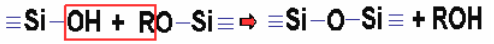
Hydrolysis



(1)

Condensation

(2)



(3)

where **R** is an alkyl group.

Eq. (1) shows the hydrolysis reaction, where alkoxide groups (OR) are replaced by hydroxyl groups (OH). Siloxane bonds (Si-O-Si) are produced by the ensuing condensation reactions involving the silanol groups. Alcohol (ROH) (Eq. (2)) and water (Eq. (3)) are also produced as by –products of condensation. Condensation (Eq. (2) and (3)) often commences before hydrolysis (Eq. (1)) is complete. Because water and alkoxysilanes are immiscible, a mutual solvent, e.g. alcohol, is used as a homogenizing agent although it is possible to prepare gels from silicon alkoxide – water mixtures without added solvent. These results were from formation of alcohol in the hydrolysis reaction, which may be sufficient to homogenize the initially phase-separated system.

Sol-gel processes obtain a lot of different inorganic and hybrid membranes, appropriated for carriers of enzymes. Sensor technique is the new area of applications of the biocatalysts.

One of the most important parameters of the biosensors is their dependence from membranes characteristics that is used for immobilizations of the biomolecules.

The response time depends on the thickness of the membrane and their surface. Operating stability of the biosensor depends of the chemical structure, kind of the membrane and the method of the biocatalysts immobilization. The linear range depends on the kinetic parameters of the enzyme: K_m, K_i, V_{max}.

For construction of the biosensors the main requirements for the membranes are: physical rigidity, chemical inertness, high photochemical and thermal stability, and excellent optical transparency.

Dong and Chen describes the biosensors, based on the new immobilization materials – sol-gel, organic-inorganic hybrid materials, cryohydrogel (or organohydrogel) and bilayer lipid membranes, are presented [9].

Since Leland C. Clark fabricated the first enzyme electrode, biosensors have been an attractive and popular field of investigations. Such devices are based on incorporating some kind of biological elements in a sensing layer intimately connected with transducer. Due to its simplicity, high sensitivity and potential ability for real-time and on-line analysis, biosensors have been widely applied in various fields including industrial process, clinical detection, and environmental control [10].

The aim of this study to develop a sol-gel method of synthesis of new hybrid membrane, with immobilized biocatalysts (microbial cells and enzymes) for biosensor construction.

2. MATERIALS AND METHODS

2.1. Synthesis of hybrid membranes – TMOS (tetramethylortosilicate) and tetraethylortosilicate (TEOS)

A new type hybrid membrane with functionalized groups was synthesized. It was based on the polymerization of inorganic precursor's tetraethylortosilicate (TEOS) or tetramethylortosilicate (TMOS), acetyl cellulose and copolymer – acrylnitrile with polyacrilamide. The syntheses of silica-based organic-inorganic polymer hybrid xerogels were carried out in ethanol solutions by combining the sol-gel and organic photopolymerisation methods.

The reagent molar ratio TEOS (TMOS): H2O was 1:16. The solution was stirred for 2.5 hour at room temperature. After 2.5 hours is added cellulose acetate to TEOS and TMOS.

TMOS and TEOS were delivered from Merck, Germany and cellulose acetate is from Aldrich, Germany.

The thin membranes were cast by spin coating method (Headway Research Inc., USA).

2.2. Immobilization of Glucose Oxidase and Peroxidase with oxidezed carbohydrate residues

The oxidation of carbohydrate residues of Glucose Oxidase /E.K.1.11.1.6/ (Merck, Germany) isolated from *Aspergillus niger* and Peroxidase /E.C.1.11.1.7/ (Merck, Germany) from horse radish with periodic acid (0.04 mM in 0.05 mM acetate buffer, pH 5.0) was performed according to the method of Zaborsky and Ogletree [11]. The unreacted periodic acid was removed with 0.025-ethylene glycol. The oxidized enzymes were dialyzed against 50 mM phosphate buffer with pH 6.0 for 18 h. The binding of the enzymes to the functionalized groups of new hybrid membranes was performed in 0.1 mM acetate buffer, pH 3.8, for 18 h at 4°C, with careful magnetic stirring. The thin membranes (TMOS or TEOS) for the individual binding of the enzymes were separately treated with 90ml enzyme solution containing 9 mg/ml glucose oxidase or peroxidase. The obtained carrier-enzyme conjugate was carefully washed with distilled water until no absorbance was observed at 280 nm in the rinsing water. The immobilized enzymes were stored at 4°C.

2.3. Construction and measurements by biosensor with immobilized glucose oxidase and peroxidase

For the construction of the biosensors, an amperometric oxygen sensor was used, as produced as Hanna instruments. The oxygen sensor of Ag/AgCl was placed in an electrolytic cell, covered with a polarizing d.c. voltage of 0-8 V. The membranes with enzymes immobilized on hybrid membranes were fixed to the surface of the oxygen electrode by means of a dialysis membrane and an O- ring.

In the case of the hybrid membrane with immobilized enzymes, a soluble polymer dextran with bound catalase was used (called by us a "liquid" membrane). Both were fixed to the surface of the oxygen electrode by a dialysis membrane and O- ring. The biosensor was dipped for each measurement, into the measuring cell, containing 0.1 mol dm^{-3} of phosphate buffer, pH 6.0 at 25°C.

The substrate was injected into the measuring cell with an automatic micropipette, the liquid in the measuring cell being continuously stirred with a magnetic stirrer.

2.4. Yeast strain and culturing condition

The *Trichosporon cutaneum* R57 strain was obtained from National Bank of Industrial Microbial and Cell Cultures, Bulgaria. The mutant basidomycete yeast strain of *Trichosporon cutaneum* have been registered by Ivanova et al. [12] under N2414. The *Trichosporon cutaneum* strain R57 cultured on a solid agar medium containing glucose, yeast extract and peptone at 28°C for 48 hours at pH 6.0. After incubation colonies were picked and suspended in a mineral salt medium with a glucose concentration of 20 $g \cdot dm^{-3}$. The composition of the nutrient medium was: $(NH_4)_2SO_4$ – 4 $g \cdot dm^{-3}$; Na_2HPO_4 – 0.75 $g \cdot dm^{-3}$; KH_2PO_4 – 1.7 $g \cdot dm^{-3}$; $MgSO_4.7H_2O$ – 0.02 $g \cdot dm^{-3}$; thiamine – 0.0002 $g \cdot dm^{-3}$ and trace mineral medium $FeSO_4.2H_2O$ – 0.001 $g \cdot dm^{-3}$; $MnSO_4.H_2O$ – 0.001 $g \cdot dm^{-3}$; $CaCl_2$ – 0.001 $g \cdot dm^{-3}$, according [13]. After 24 h incubation in a bath shaker at 28°C, pH 6.0, the cells were suspended in the same nutrient medium containing hybrid membrane TMOS under the same conditions [14].

Periodically the matrixes were washed up by physiological solution and suspended in the fresh nutrient medium.

By scan electron microscopy was studied the formation and development of biofilm from *Trichosporon cutaneum* R57. The pictures are done on 6^{th} and 26^{th} day of the biofilm development.

3. RESULTS AND DISCUSSION

3.1. Characterisation of bond enzymes

On the Table 1 are presented the kinetic parameters of investigated enzymes: glucose oxidase and peroxidase.

Table 1. Kinetic parameters of enzymes

Enzymes	Membranes	Specifically activity [U/mg]	Relative activity [%]	Amount of bound protein	pH_{opt}	T_{opt} [°C]
Glucose oxidase	-	6.47	-	-	6.0	30
Immobilised glucose oxidase	TMOS	5.83	90.11	1.42	6.0	30
	TEOS	2.36	36.48	1.25	6.5	35
Peroxidase	-	33.88	-	-	6.0	35
Immobilized peroxidase	TMOS	19.39	57.23	1.09	6.0	35
	TEOS	10.69	31.55	0.74	6.5	30

How it has been seen from the table 1 that the pH and T°C optimum are changed for immobilized enzymes on hybrid membrane containing TEOS.

3.2. Construction of biosensors using TMOS membrane with covalently immobilized glucose oxidase

The measurements are done by amperometric biosensor according to part “Materials and Methods”. On the Fig. 1 (a, b) are shown the response time curves in the different concentrations of glucose.

The plots show that the stability of biosensor is achieved for 15-60 seconds, depends of the substrate concentrations. The operating stability was biosensor is 20 days.

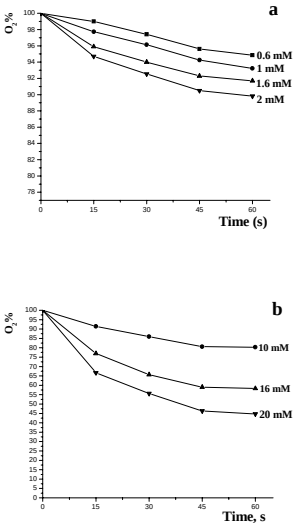


Fig. 1. Response time of the biosensor containing hybrid membrane with immobilized glucose oxidase, at low substrate concentrations (a) and at high substrate concentrations (b).

The main parameters of amperometric biosensor are represented on Table 2.

Table 2. Parameters of biosensor with hybrid membrane

Matix	Substrate	Linear range [mM]	Correlation index	Operating stability	Response time [s]
Hybrid membrane [TMOS]	Glucose	0.6 – 20mM	0.99227	20	15-60

3.3. Formation of biofilm from Trichosporon cutaneum R57

Formation of biofilm is done according to "Materials and Methods. Producing of extracellular polymeric structure (EPS) and development of biofilm is visualized by scanning electron microscopy (JEOL JSM- 5510, JEOL JFC- 1200 fine coater).

On the Fig. 2 is represented the blank hybrid membrane containing TMOS. The surface of the membrane is homogenous with pores<1μm. On the Fig. 3 and Fig. 4 is shown the allocation of microorganisms on the matrixes of the 6th day and 26th day respectively of biofilm formation. The producing of extracellular polymeric substances (EPS) is studied in the time. On the 26th day of biofilm formation, EPS almost cover the surface of the membrane. Fig. 5 shows the yeast cell in the moment of EPS secretion.

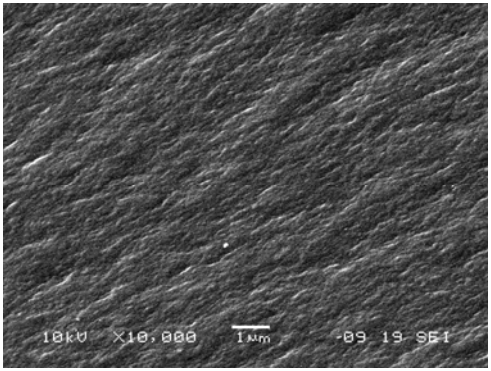


Fig. 2. SEM of blank hybrid membrane

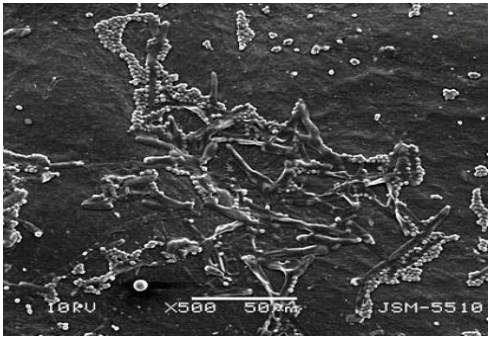


Fig. 3. SEM images of formation of biofilm after 6 days

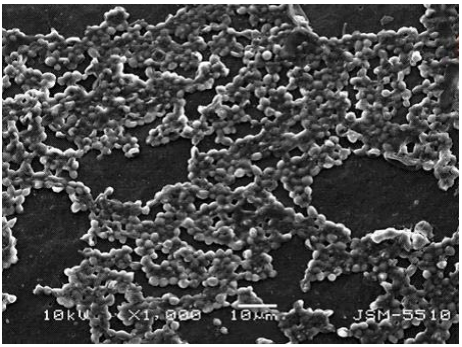
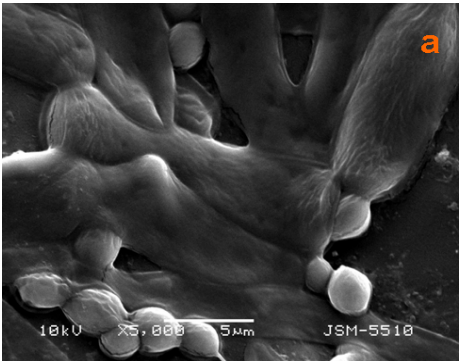


Fig. 4. SEM images of biofilm formation after 26 days



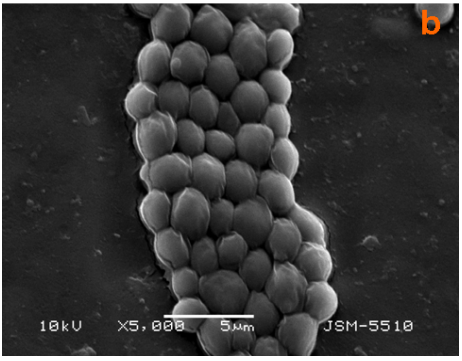


Fig. 5. SEM images of EPS
from *Trichosporon cutaneum* R57 (a, b)

The producing of EPS from the cells on the membranes surface is a proof for biological compatibility between the cell and carrier and for optimum conditions of structure development. Extracellular polymeric structures producing by the yeast, cells have oval shape and thick arrangement. The size of every one unit is almost 1μm, but the size of the *Trichosporon cutaneum* R57 is (2 - 15)x(10 - 15)μm. From Fig. 5b it can be seen the specific allocation of the EPS, as their shape, size and arrangement is individual for every kind of microorganisms which producing EPS [15].

4. CONCLUSIONS

This study shows that hybrid organic-inorganic membranes were synthesized. There were used precursors-TEOS and TMOS for SiO_2, cellulose acetate and co-polymer between acrylonitril and acrylamid. Covalently immobilized glucose oxidase contains 90.11% (TMOS), 36.48% (TEOS) and covalently immobilized peroxydase contains 57.23% (TMOS), 31.55% (TEOS). Biosensor containing hybrid membrane for glucose register wide working range 0.6-20 mM, short response time – 45 s and good reproduction – 10 days. Hybrid membranes are appropriate for formation of biofilm from strain *Trichosporon cutaneum* R57.

The future work will be concentrated in these investigation fields:

A biosensor consisting of immobilized yeast cells *Trichosporon cutaneum* R57 and an oxygen probe will be developed for BOD estimation. Yeast cells will be immobilized on different hybrid membranes and will be trapped between the pores of Teflon membranes. The major interest is the pesticides biosensors based cholinesterase for analysis of organophosphate and carbamate compounds. These devices have found application also in acetylcholine determination, a biomolecule that plays an important role in nerve impulse transmission in the peripheral and central nervous system. The advantages of these biosensors are their higher sensitivity, short response time, high stability, no sample purification is required, and so cell suspensions and whole blood can be used.

Acknowledgements
The present work is funded and supported by project No R-13, 2005 of Ministry of Education and Science.

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