Invited Paper

Constructing Optical Biosensor Based on Multienzyme System Tyrosinase/Horseradish Peroxidase

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***Abstract*:** *By using sol-gel method, hybrid membranes were synthesized based on SiO_2 and cellulose derivatives. Membranes showed good physical and mechanical properties as well as good biocompatibility. Onto hybrid membranes tyrosinase and horse radish peroxidase were immobilized and optical biosensor was constructed. The working principle of constructed biosensor was detection of oxygen consumption. The properties of constructed biosensors like linear range, life time and sensitivity were defined by modeling substrate – L-3,4-dihydroxyphenylalanine. The factors affecting the constructed optical biosensors were investigated. The results showed that a multienzyme system is capable of improving the constructed biosensor properties.*

Keywords: *Optical biosensors, Enzymes, Sol-gel, Toxic compounds detection.*

Introduction

Optical biosensors are powerful alternative to conventional analytical techniques, particularly for their high specificity, sensitivity, small size, and cost effectiveness. The research and technological development of optical biosensors have experienced an exponential growth during the last decade because this technology has a great potential for direct, real-time and label-free detection of many chemical and biological substances [5]. Fig. 1 shows the year on year development of biosensor market [8] and scientific publications [1].

The advantages of the sol-gel technology are undoubtedly simplicity and versatility. It enabled to obtain for example oxides in the form of layers, powders, monoliths or fibers. These materials are successfully applied for sensing purposes due to their properties such as transparency, porosity and high surface areas [12].

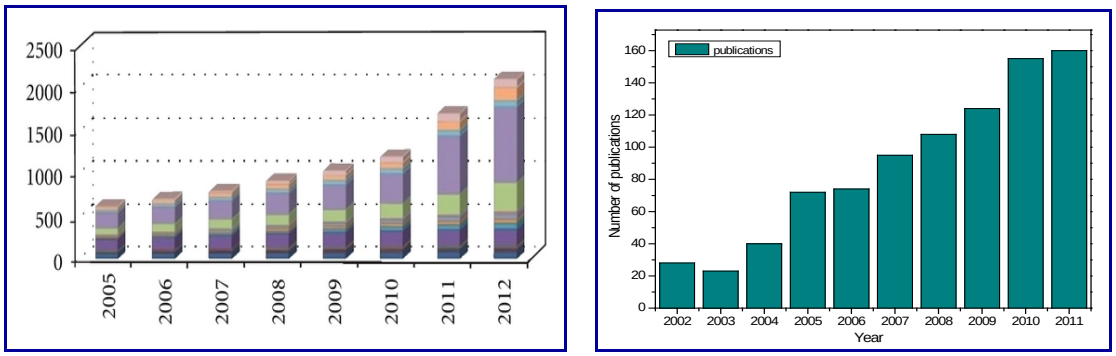

Fig. 1 Graph showing the development of optical biosensor in the last decade [8] (left side) and scientific publications (right side) [1]

Tyrosinase (EC 1.14.18.1) is a type of catechol oxidase and contains a binuclear copper centre. The reaction catalyzed is as follows:

$$\text{L-tyrosine} + \text{L-DOPA} + O_2 \rightarrow \text{L-DOPA} + \text{dopaquinone} + H_2O.$$

It has two binding sites, the substrate binding site which has an affinity for aromatic compounds, and the oxygen site which has an affinity for coordinating agents that bind to the metal. The enzyme catalyses the hydroxylation of monophenols to *o*-phenols and the oxidation of *o*-phenols to *o*-quinones. Tyrosinase is inhibited by many different compounds such as carbamate and dithiocarbamate pesticides, atrazines, chlorophenols and thioureas and these characteristics have been used to develop biosensors for the enzymatic detection of many pesticides and other toxic compounds indicated. Tyrosinase biosensors suffer from poor specificity and many substrates and inhibitors interference with the enzyme's ability to detect the target compounds. The enzyme is further inherently unstable, reducing the lifetime and usability of the tyrosinase-based biosensors. Tyrosinase has a higher tolerance for organic solvents and biosensors have been constructed that operate with an organic phase. A tyrosinase biosensor can also be operated fast [16].

Many biosensor research papers have been reported previously for the detection of phenolic compounds based on several types of enzyme such as tyrosinase [3], laccase [10], horseradish peroxidase (HRP) [4]. However, reaction mechanisms of the biosensors based on tyrosinase, laccase and HRP are different for various types of phenolic compounds. For tyrosinase and laccase, the enzyme molecules are reduced by phenolic compounds after being oxidized by oxygen. For HRP, it is oxidized by hydrogen peroxide after its reduction by phenolic compounds.

Materials and methods

Tyrosinase isolated from mushrooms (E.C. 1.14.18.1) and peroxidase isolated from horseradish (E.C. 1.11.1.7) were purchased from Sigma-Aldrich; L-3,4-dihydroxyphenylalanine (L-DOPA) from Fluka triethoxysilane (TEOS), trimethoxysilane (TMOS) methyltrietoxysilane (MTES), ethyltrimethoxysilane (ETMS) from Merck; cellulose acetate propionate with high molecular weight (CAP/H)), ~25 000 and cellulose acetate propionate with low molecular weight (CAP/L) and 15 000, respectively, from Sigma-Aldrich; co-polymer of acrylamide/acrylonitrile (AA) was provided by the Biotechnology Department of University of Chemical Technology and Metallurgy, Sofia, Bulgaria; dimethylformamide (DMF) from Merck.

Synthesis of hybrid membranes

On Fig. 2 is presented schematic illustration of hybrid membranes synthesis. Per each membrane amount of compounds are: 5 ml for silica precursors; 10 ml ethyl (for TEOS and MTES) or methyl (for TMOS and ETMS) alcohol, 3 g cellulose derivatives (with low or high molecular weight) dissolved in chloroform, 100 mg co-polymer of acrylamide/acrylonytrile.

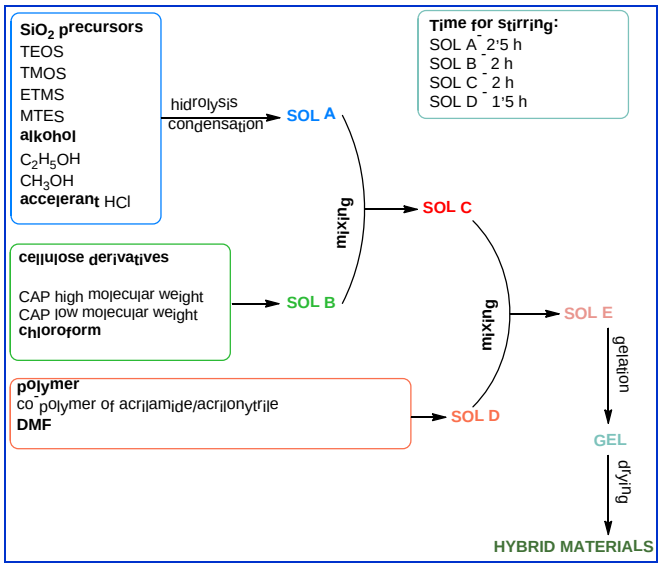

Fig. 2 Schematic illustration of hybrid membranes synthesis

Oxidation method for tyrosinase and peroxidase

Oxidation of the carbohydrate residues of tyrosinase was done with periodic acid according to Zaborsky and Ogletree's [18] method. The oxidized enzyme was dialyzed in a dialysis membrane from Serva, Germany, by submerging for 24 h in 50 mmol/L phosphate buffer with pH = 6.0.

Determination of enzyme activity and content of protein

The reaction mixture containing tyrosinase was aerated with Vortex, BOECO. L-DOPA was used as a substrate for determination of percent of oxygen consumption (%) by tyrosinase/peroxidase multienzyme system. The total protein content in the immobilized enzymes was determined by the Lowry [11] modified method using bovine serum albumin as a standard.

Constructing an optical biosensor for detecting the consumption of oxygen (%)

The optical biosensor was constructed using the membrane with the best parameters. The membranes were prepared by the method of spin coating (Headway Research Inc., USA), at 2000 rpm and 100 s of drying. On the membranes obtained in this manner, co-immobilization of tyrosinase and peroxidase was done under continuous stirring for 16 h at t = 4 °C. The measurements were done using spectrophotometer and oxygen probe of (Avantes Spec 2048 Co. Inc). Oxygen probe is based on the quenching effect of the oxygen on the fluorescence of the ruthenium complex. The AvaLight-LED-475 light source used to emit light with a wavelength of 475 nm. Measurement of the fluorescence is performed at 600 nm. Using oxygen probe of Avantes fiber optic device, the membrane with immobilized enzymes tyrosinase and horseradish peroxidase was fixed onto the surface of the probe. The consumption rate of oxygen was used as an indicator of enzyme activity. After every measuring the electrode was washed with distillated water.

Results and discussion

The cellulose derivatives are excellent natural biomaterials. During the sol-gel process, the inorganic material, such as TEOS, is deposited within the cellulose matrix forming hydrogen bond between organic and inorganic phases [17].

After oxidation of the carbohydrate moieties, HRP was covalently immobilized onto hybrid membranes. Fig. 3 presents the amounts of bounded protein onto various carriers.

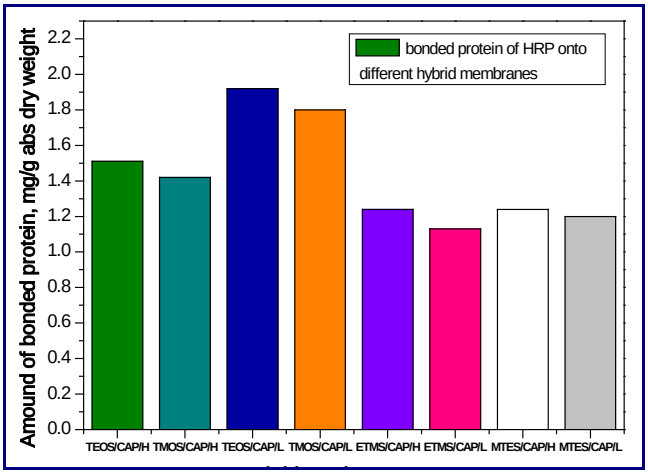

Fig. 3 Amount of bonded protein onto different type of hybrid membranes

The results of the graph show the highest levels of the enzyme linked to the membranes containing the TEOS/CAP/H, TMOS/CAP/H, TEOS/CAP/L and TMOS/CAP/L. The results obtained for the specific and relative activity of the membrane containing the TEOS/CAP/H, TMOS/CAP/H, TEOS/CAP/L and TMOS/CAP/L proved unsatisfactory. Despite the high values of the bound enzyme was observed and relatively low specific activity. The reason for this can be a heterogeneous structure and the presence of large amounts of aggregates with various sizes, which prevents uniform penetration of the substrate into the membrane. Possible diffusional limitations of the matrix are large quantities of components in the structure. The results obtained for the diffusion limitations corresponded to those reported by Bayramoglu and co-authors [2].

Figs. 4-6 show the dependence of oxygen consumption on different substrate concentration. An increase in the concentration of the substrate results in an increases in oxygen consumption. Based on these graphs, the best membrane that makes more consumption of oxygen among the membranes is the membrane that contains cellulose acetate propionate with high molecular weight. This means that the enzyme immobilized onto this membrane shows good kinetic parameters.

Table 1. Kinetic parameters for oxygen consumption of biosensors based on hybrid membrane

Membrane	K_m **(O_2), µM**	V_{max} **(O_2), mg O_2/L/min**	R^2
ETMS/CAP/H/	98.54	1.27	0.9693
ETMS/CAP/L/	93.91	1.25	0.8995
MTES/CAP/H/	133.12	1.37	0.9224
MTES/CAP/L/	344.1	0.95	0.8339
Free tyrosinase	142.7	2.18	0.9966

The results presented in Table 1 show that all hybrid membranes have similar properties. The results from kinetic parameters corresponded with results reported by Ha and co-authors in [7, 13] and [9].

The statistical analyses of the samples are shown in Tables 2-4. The values of the mean, standard deviation, variance and the relative standard deviation % after measuring the oxygen consumption for 10 consecutive samples were calculated. Results for mono-enzyme and multienzyme systems are compared by free tyrosinase.

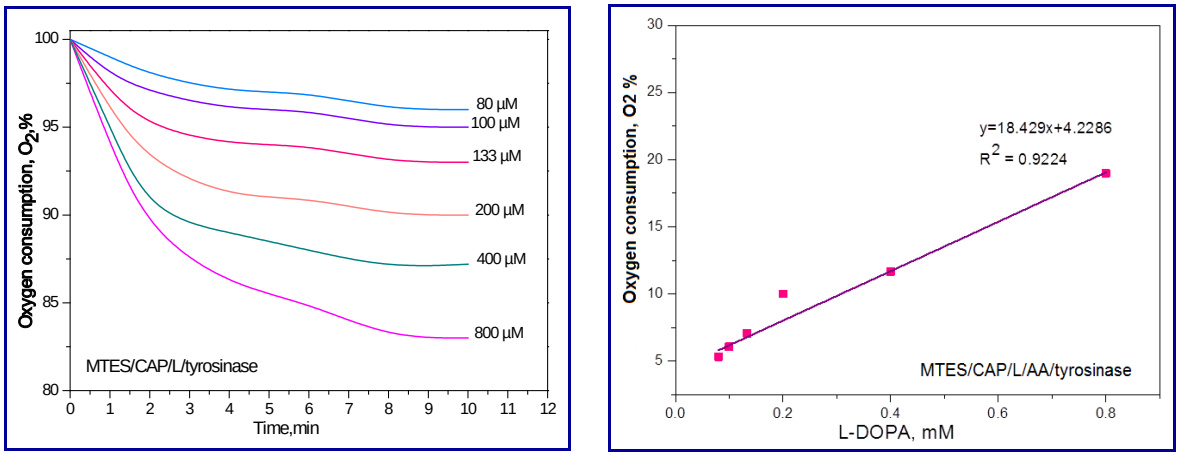

Fig. 4 Response time of the optical biosensor based on MTES/CAP/L/tyrosinase at different L-DOPA concentrations and calibration curve of L-DOPA

Table 2. Statistic data of the oxygen consumption (O_2, %) measurements obtained by the biosensor constructed by hybrid membrane MTES/CAP/L/tyrosinase

MTES/CAP/L/AA/tyrosinase						
Concentration L-DOPA	***n***	**Mean ($\bar{X}$)**	**Standard deviation (*S*)**	**Coefficient of variation**	**R.S.D. (%)**	**Variance**
80 µM	10	96.53	0.01938	0.02008	2.01	0.000376
800 µM	10	84.17	0.02192	0.02604	2.60	0.021925

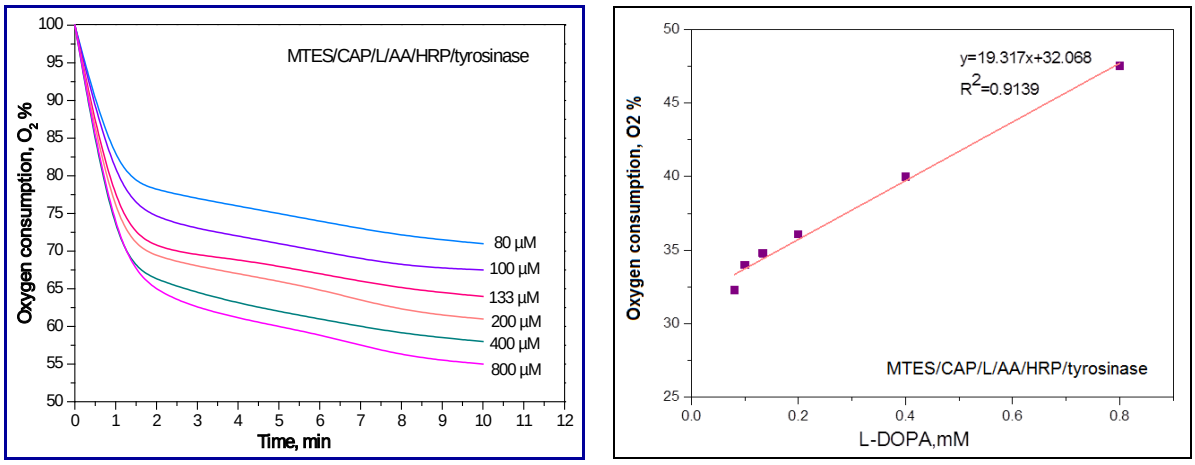

Fig. 5 Response time of the optical biosensor based on multi enzyme system at different L-DOPA concentrations and calibration curve of L-DOPA

Table 3. Statistic data of the oxygen consumption (O_2, %) measurements obtained by the biosensor constructed by hybrid membrane MTES/CAP/L/HRP/tyrosinase

MTES/CAP/L/HRP/tyrosinase						
Concentration L-DOPA	***n***	**Mean ($\bar{X}$)**	**Standard deviation (*S*)**	**Coefficient of variation**	**R.S.D. (%)**	**Variance**
80 µM	10	71.06	0.01230	0.017311	1.73	0.000151
800 µM	10	55.15	0.00999	0.01812	1.81	0.00009

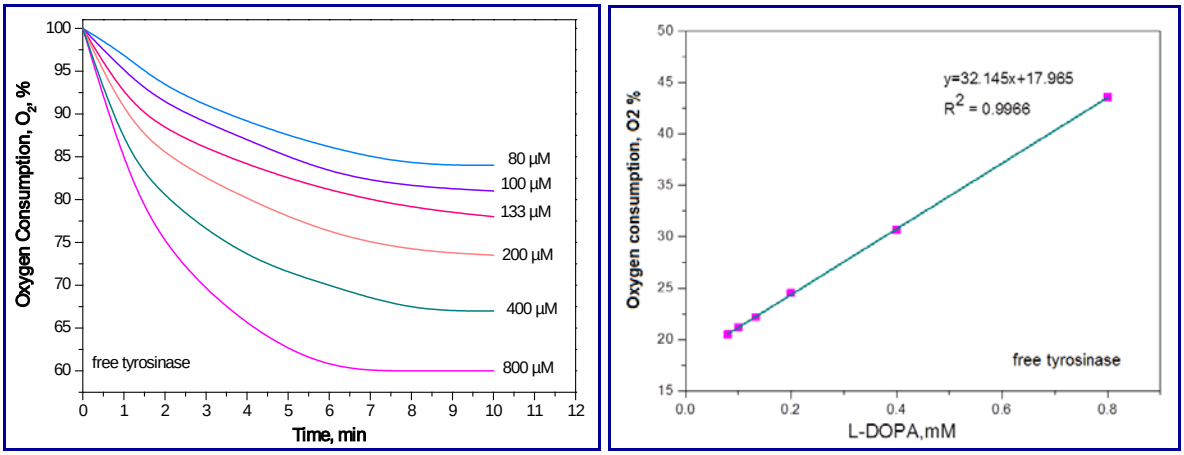

Fig. 6 Response time of the free tyrosinase at different L-DOPA concentrations and calibration curve of L-DOPA

Table 4. Statistical data of the oxygen consumption (O_2, %) measurements of free tyrosinase

Free tyrosinase						
Concentration L-DOPA	***n***	**Mean ($\bar{X}$)**	**Standard deviation (*S*)**	**Coefficient of variation**	**R.S.D. (%)**	**Variance**
80 µM	10	83.92	0.002939	0.00350	0.35	0.00008
800 µM	10	59.86	0.002276	0.00380	0.38	0.00005

It was observed that the multi-enzyme system allows a good reproducibility of the optical biosensor using spectrophotometer and using oxygen probe. It was observed that the coefficient of variation (R.S.D. = 1.81%, n = 10) for 800 µM L-DOPA after measuring the oxygen consumption. These results are close to the results obtained by Teixeira et al. [15] and Nezhda et al. [13]. They demonstrated reproducibility (R.S.D. = 3.7%, n = 9) and (R.S.D. = 2.5%, n = 6) respectively. Applying multienzyme system tyrosinase/HRP enhanced the catalytic activity of the enzyme, and improved operating parameters of the biosensor. There is an average of 33% increase in oxygen consumption for all tested matrices even at low substrate concentrations [14]. Design of optical biosensor measuring oxygen consumption based on tyrosinase/HRP showed two times higher oxygen consumption for the substrate L-DOPA, in comparison with the optical biosensor with immobilized tyrosinase. Design optical biosensor measuring oxygen consumption based on covalently immobilized tyrosinase shows a wide linear range of action of 0.8×10^{-5}-8×10^{-5} M for L-DOPA, short response time, good reproducibility study period of 30 days, and correlation coefficient the standard rules R^2 = 0.9224 as a carrier MTES/CAP/L/AA.

Conclusion

The fiber-optic biosensor based on simultaneous covalent immobilization of tyrosinase and horseradish peroxidase onto silica hybrid membrane for toxic compounds detection was constructed. The designed optical biosensor based demonstrated high stability, short response time and wide linear working range of the L-DOPA (80-800 μM). This biosensor can be potentially applied in the analysis of food, cosmetics and medical diagnostics, as well as monitoring of the environmental pollutants.

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