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Design of optical biosensors for detection of pharmaceutical products

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Sol–gel approach has rapidly become a fascinating new field of research in materials science. 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.

The aim of our work is a design of optical biosensors for detection of pharmaceutical products. We synthesized by sol-gel methods hybrid matrices contained silica precursors, cellulose derivatives and Poly (amido amine) dendrimers (PAMAM) as perspective carriers for covalent immobilization. Horseradish peroxidase (HRP) was used as a model enzyme. Conditions were optimized, kinetic parameters, pH and temperature optimums were determined. Constructed biosensors were implemented to detect resorcinol, pirogallol, epinephrine and etc.

These biosensors can be potentially applied in medical, pharmaceutical, food and environmental monitoring fields.

Key words: optical biosensors, hybrid materials, toxic compounds detection.

INTRODUCTION

Demands for developing biosensors like amperometric [1], potentiometric [2] and optical sensors [3] for controlling the quality of food [4], pharmaceuticals [5], environmental activities [6, 7] and industrial processes [8] have encountered an enormous increase over the last decade. A lot of effort was devoted to design highly sensitive and selective methods for that purpose [9, 10]. Biosensors are self-contained analytical devices that associate a biological sensing element and a transducer to respond selectively to chemical species in the monitored samples and are considered a powerful tool compared to the existing traditional techniques; this is because of their high specificity, efficiency, direct use, reliability and cost effectiveness. [11, 12, 13]

Using enzymes as a promising biological sensing element because of their specificities is limited by their low stability, low availability and high cost. Immobilizing enzymes enhances their stability [14, 15, 16], reduces the required amounts by recovering them [3] so optimizes operational costs, while maintaining activity and signal of detection [17, 14].

Using peroxidases to catalyze oxygenation of different substrates in presence of hydrogen peroxide was reported [18, 19]. Horseradish peroxidase (HRP) was used for analytical purposes [20] to produce signals that could be based on colorimetry [15], chemiluminescence, fluorescence [21] or amperometric measurements [1].

An optical biosensor was developed by covalent immobilization of horseradish peroxidase onto prefabricated matrices. Matrices were constructed by sol-gel method; to be mainly composed of cellulose acetate butyrate (CAB) and Poly (amido-amine) (PAMAM) dendrimers as the organic part and prepared SiO_2 nanoparticles with precisely controlled size as the inorganic part that were optimized and mixed for developing the matrices to construct the optical biosensor. The system response to substrate, catalytic properties and other parameters like pH, temperature, sensitivity and stability were examined.

EXPERIMENTAL

Reagents

Peroxidase isolated from horseradish (E.C.1.11.1.7) purchased from Sigma–Aldrich; trimethoxysilane (TMOS) from Merck; cellulose acetate butyrate

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(CAB), Polyamidoamine (PAMAM) dendrimers second generation from Sigma–Aldrich; phenol, hydrogen peroxide solution (35%) and chloroform from Merck. Other chemicals were of analytical reagent grade. Double-distilled and deionized water was utilized.

Synthesis of hybrid matrices by sol-gel method

Preparation was done via two separate steps, by separating the organic part preparation (PAMAM/CAB) and inorganic component (SiO_2). This was followed by optimized mixing of the two components to prepare the matrices.

Methods

Transmission electron microscopy (TEM). High Resolution Transmission Electron Microscope: JEM-2100 with 200kV/ Cu grids was used for characterizing the size and morphology of prepared SiO_2 nanoparticles used within the study.

Fourier transform-infrared (FTIR) Spectroscopy. Structure and covalent immobilization were investigated using JASCO FTIR-5300, on free HRP, free matrices, TMOS/CAB/PAMAM/A and TMOS/CAB/PAMAM/B.

SiO_2 nanoparticles preparation. SiO_2 were synthesized by the modified Stöber method; 0.6 mL of trimethoxysilane (TMOS) was hydrolysed and condensed to give SiO_2 in two mixed alcohol solutions of ethanol and nethanol (A and B) with a basic catalyst of ammonia solution (1 mL of water and 3 mL of 30% NH_4OH in 50 mL of total alcoholic solution used). The relative volume ratio of methanol: ethanol was 8:1 (a) and 4:1 (b) while all the other factors were fixed.

Cellulose acetate butyrate (CAB) was used as the organic component, with PAMAM dendrimers as a source of active groups for covalent immobilization of HRP as being done before by our team [22].

Chloroform was used as a solvent for CAB, 50 µl PAMAM dispersed in chloroform was added, and then the mixture was stirred at room temperature for 3 hours.

Previously prepared SiO_2 particles were added in a percentage of 3% of the total cellulose acetate butyrate added. Mixture was then stirred well followed by membrane casting in the desired shape and drying at room temperature to induce phase separation yielding a microporous structure [23].

Oxidation of HRP. Oxidation of carbohydrate residues of horseradish peroxidase was done according to Zaborsky and Ogletree's procedure [24]. The oxidized HRP was dialyzed by dialysis membrane from Serva, Germany, through submerging for 24 hours in 50 mmol/L acetate buffer with pH 5.6. Conditions utilized for oxidation of carbohydrate residues of HRP and that for immobilization were used to preserve HRP activity and having high immobilization efficiency [25].

Covalent immobilization of peroxidase onto hybrid matrices. HRP covalent immobilization onto matrices was done using Zaborsky and Ogletree approach, via covalent linkage between amide groups flanking onto the prefabricated matrices and the carbohydrate residues of HRP [24]. HRP covalent immobilization was carried out as follow: 1.0 g of the hybrid matrices was added to 10 ml of the oxidized dialyzed solution of HRP. Immobilization was done by continuous stirring for 24 h at 4 °C.

Determination of enzyme activity. HRP activity was measured spectrophotometrically by measuring the increase of absorbance (λ = 510 nm) for the solution of oxidized 4-APP (4-aminoantypyrin), as being converted to the reduced form by HRP. The assay was done within phosphate buffer (PBS, 100 mM, pH 6.5) having 4-APP (1.4 mL/2.5 mM), H_2O_2 (1.4 mL/1.7 mM), at 25 °C.

Total protein determination. Total free and immobilized protein was determined via modified Lowry method taking bovine serum albumin as a standard [26].

pH and temperature optimum

The optimum operational pH for immobilized HRP was determined using 100 mM phosphatic buffer within the pH range 4.0–8.5 using 100 µM solution of hydrogen peroxide. Temperature optimum was determined in the range from 20 °C to 65 °C.

Drying time (Drying characteristics). This is done by recording the amount of evaporated solvent from the system with respect to time. Matrices were weighed immediately after the application of the coating, and then the weight loss was recorded until constant weight was reached.

RESULTS AND DISCUSSION

The constructed hybrid matrices exhibited enhanced uniformity, flexibility, transparency and surface properties, which is desirable properties for sensing applications and for enzyme immobilization. Both used organic and inorganic exhibits biocompatibility, while matrices exhibit inertness towards the enzyme, in addition to the low preparation cost, simplicity, and versatility of the used sol-gel process [25, 27].

Figure 1 shows the Transmission Electron Microscopy (TEM) of SiO_2 nanoparticles, SiO_2 nano-

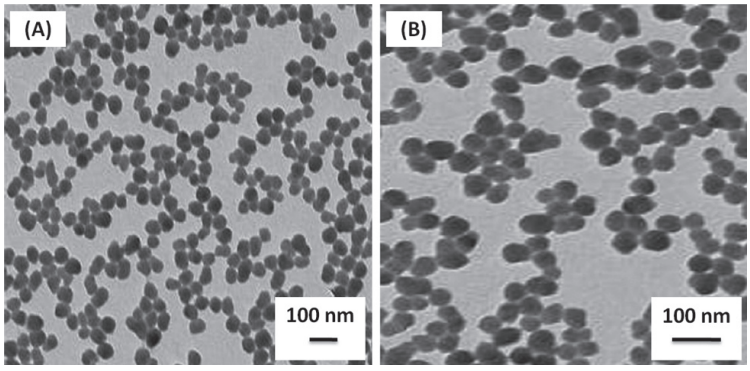

Fig. 1. TEM images of SiO_2 particles prepared in the mixtures of methanol: ethanol (v/v); (a) 8:1, and (b) 4:1

particles appeared to be of spherical shape, with estimated average size around 50 nm and 65 nm. It is believed that the changes of the size of SiO_2 particles in the mixed alcohol solvent might due to the average effect of polarity, hydrogen bonding and viscosity of two used alcohols [28].

Optimized addition of silica nanoparticles around 3% contributes in enhancing mechanical properties while the integrated silica within the cellulosic matrix forms hydrogen bonding and so founding a connection between both organic and inorganic phases while decreasing the internal pressure gradients during membrane drying [29]. Using PAMAMs which is a polymeric material is believed to affect the inorganic condensation-polymerization process and that of the sol-gel material [30].

Fourier transform infrared spectroscopy studies

As shown by FT-IR measurements for free HRP, matrices before immobilization, TMOS/CAB/PAMAM/A and TMOS/CAB/PAMAM/B (Figures 2a, b, c and d respectively), the sharp band

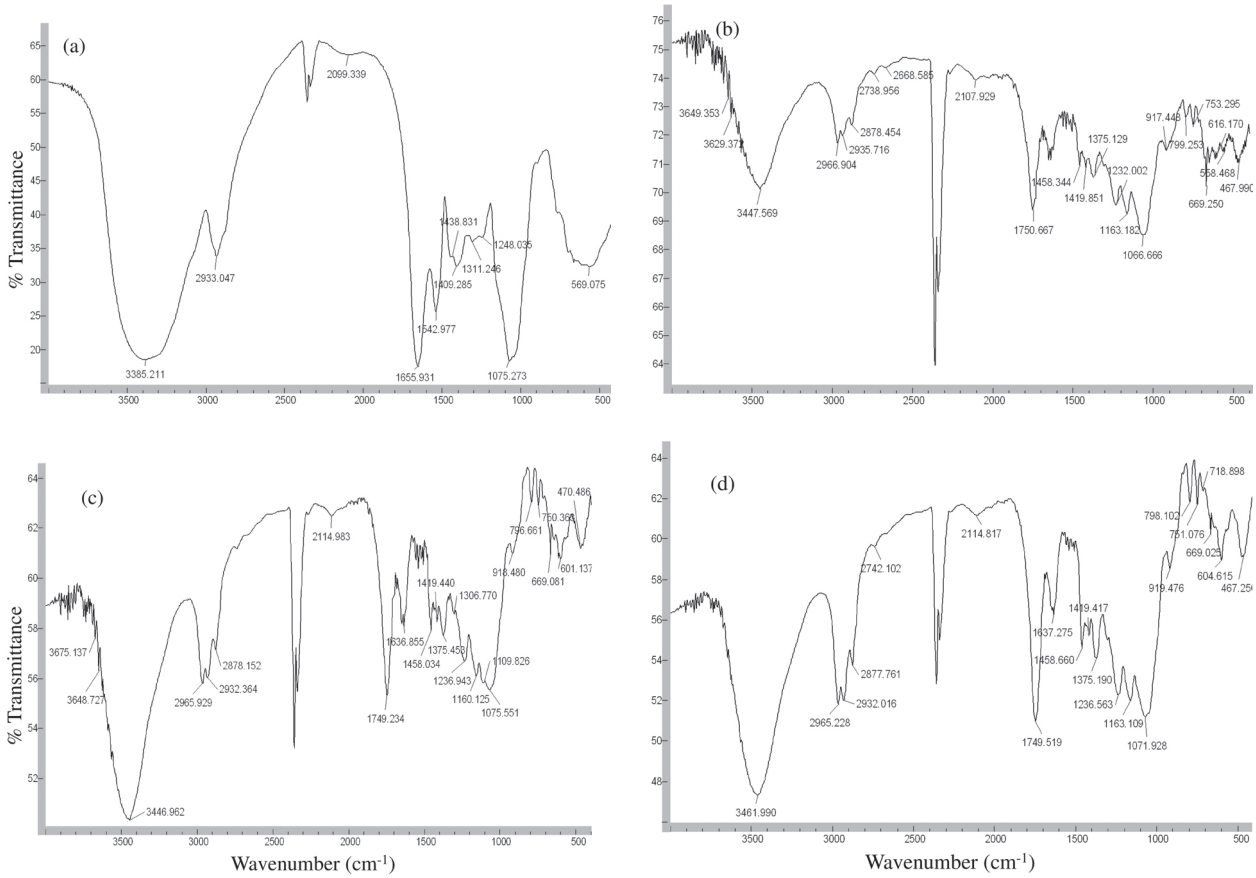

Fig. 2. FT-IR (JASCO FTIR-5300) spectrum (a) Free HRP, (b) Only matrices, (c) TMOS/CAB/PAMAM/A and (d) TMOS/CAB/PAMAM/B

Table 1. Catalytic properties of free and immobilized HRP

Sample	Abs. dry wt. [mg/g]	Specific activity [U/mg]	Relative activity [%]	pH optimum	Temp. optimum [°C]
Free HRP	–	31.7		6	30
TMOS/CAB/PAMAM (A)	2.48	29.8	94	6.5	40
TMOS/CAB/PAMAM (B)	2.5	28.7	90	6.5	45

at 1071 cm^{-1} corresponds to Si-O-Si stretching vibrations, indicating SiO_2 existence within matrices. The appearance of a broad peak within the range of 3100–3550 cm^{-1} might be corresponding to the -NH- of HRP protein, beside the clear increase within the intensities of the sharp peaks at 1637 cm^{-1}, 1458 cm^{-1}, and 1236 cm^{-1} that is attributed to amide I (–CONH–) (the C=O stretching vibration of the peptide linkage in the protein background), amide II and C–N stretching of the amide III respectively. Appearance and enhancements of these peaks may be also attributed to the HRP protein, so matrices with immobilized HRP displaying these bands, indicating successful covalent immobilization of HRP.

Catalytic properties of free and immobilized HRP

As shown in Table 1, catalytic properties for immobilized HRP showed high values compared to the free HRP. This goes with previous reports about CAB matrices with its high surface area and open structure at the end [31]. TMOS/CAB/PAMAM (A) showed better catalytic properties, with the highest relative activity recording 94%, while TMOS/CAB/PAMAM (B) relative activity was 90%. On the other hand, under pH 7.0 TMOS/CAB/PAMAM (A) and TMOS/CAB/PAMAM (B) recorded close levels of the bounded HRP recording 2.48 mg and 2.5 mg bounded protein per gram absolute dry weight respectively.

Enhanced catalytic properties and amount of bounded protein might be due to optimized addition of SiO_2 nanoparticles; SiO_2 act as a surface modifying molecules that optimized the formation of more graded pore sublayer structure with higher porosity which resulted in an enhanced permeation rate through the membrane [33, 32], providing more surface area for higher quantity of HRP immobilization and better substrate penetration into the membrane and the products out by decreasing substrate diffusion limitation [34] as well as offering higher protection from the environment, explaining the resulted increase in the activity [35].

Table 2 is comparing kinetic parameters of immobilized HRP against parameters of free HRP.

Table 2. Kinetic parameters for free and immobilized HRP onto hybrid matrices

Sample	Km [μM]	Vmax [μM/min]	R^2
Free HRP	0.96	18.5	0.998
TMOS/CAB/PAMAM (A)	3.24	27	0.997
TMOS/CAB/PAMAM (B)	0.94	13	0.971

Using Lineweaver-Burk plotting for characterization of our immobilized HRP allowed calculation of apparent Michaelis constants and maximum reaction velocity.

Immobilized and free HRP exhibited response to the reduction of phenol concentration with a recorded range from 0.09 mM/L till 2 mM/L for phenol. For immobilized and free HRP Km values obtained were found varying between 0.94×10^{-3} and 3.24×10^{-3}. Kinetic parameters results corresponded with results reported by Mohamed and co-authors [36].

Figure 3 shows the dependence of the steady state of immobilized HRP on the concentrations of phenol, the increase in response was linear up

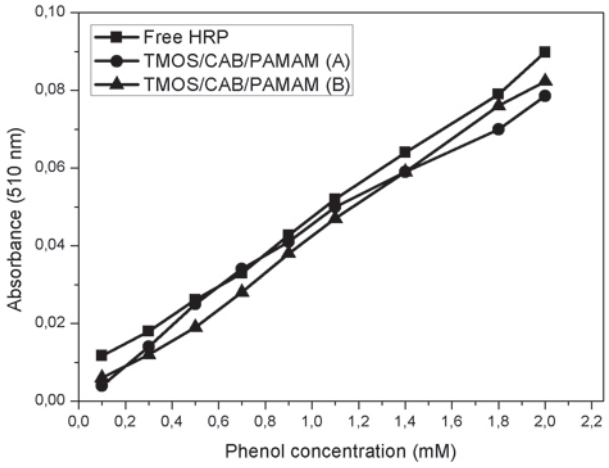

Fig. 3. Dependence of the absorbance on concentration of phenol

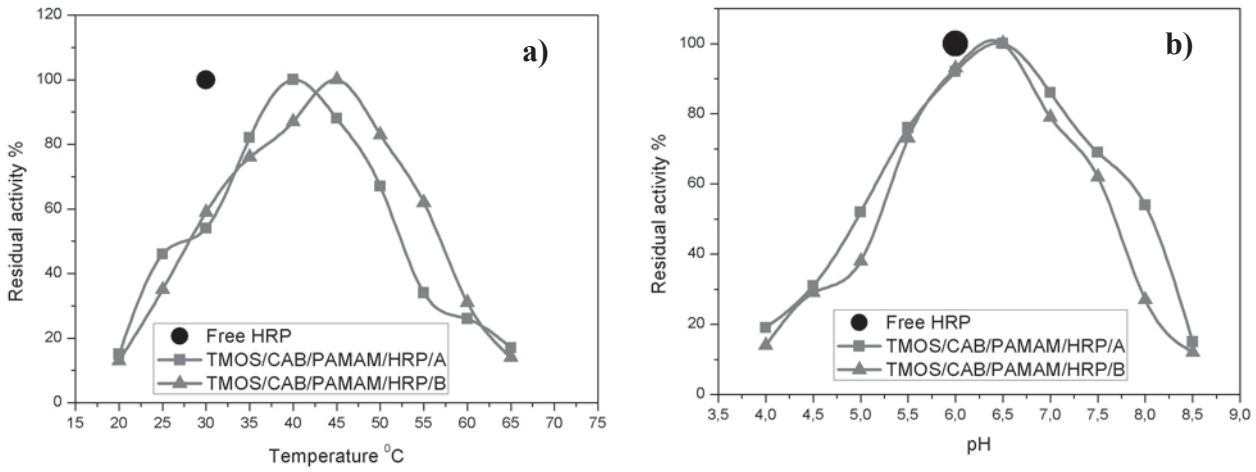

Fig. 4. (a) Temperature effect on residual activity of Immobilized HRP at pH 6.0; (b) pH effect on residual activity of HRP at room temperature

to 2.0 mM/L for free HRP, 1.4 mM/L for TMOS/CAB/PAMAM (A) and 2 mM/L for TMOS/CAB/PAMAM (B). Results coincide with previously reported data by Arzum et al [1].

Figure 4 (A) shows the residual activity of free and immobilized HRP as a function of temperature, results showed higher thermal stability for immobilized HRP onto the two matrices through shifting of temperature optimum from 30 °C for free HRP to 40 °C for TMOS/CAB/PAMAM/HRP/A, and 45 °C for TMOS/CAB/PAMAM/HRP/B.

Matrices were believed to provide better environment and protection for HRP that affected temperature optimums and enzyme stability, These observations corresponds with work done by Yotova et al [15].

Figure 4 (B) shows the optimum operational pH, pH shift was recorded for the two matrices having 100% residual activity around pH 6.5, compared with optimum pH for free HRP that was around 6.0. Immobilized HRP curves showed relatively high residual activity over a wider pH range, which goes with previously reported results [37].

After the measurements were done, the matrices with the immobilized HRP were stored in the phosphate buffer solution (0.01 M, pH 6.0) at 4 °C. As shown in Figure 5 (a), results show that immobilized HRP retained about 80% of the initial enzyme activity after 10 successive cycles of application. the other hand, Figure 5 (b) shows that the system with Immobilized HRP retained about 85% of the initial activity 60 days after enzyme immobiliza-

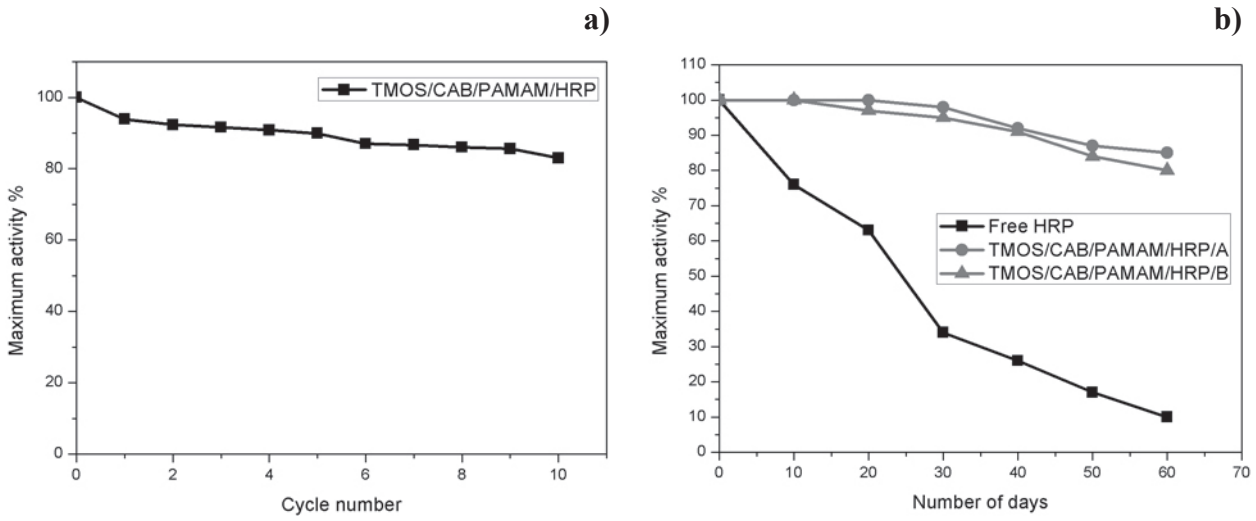

Fig. 5. Maximum activity retained versus (a) number of cycles, (b) storage period

tion compared to the free HRP that retained about 10–15% after this period.

Drying behavior

Drying process is considered critical step in membrane casting which significantly affects the final membrane morphology [38]. On solidification polymer rich phase precipitated forming solid matrix that includes the polymer phase which is rich in solvent [39]. In general drying membranes using higher temperature can leads to shrinkage of the surface pore size, and as cellulose acetate membranes demonstrates low resistance to shrinkage it was important to optimize the drying step.

The silicate nano-fillers used had an impact on accelerating the drying process while avoiding shrinkage and deterioration of matrices; Drying was accomplished at 25 °C overnight with final residual solvent less than 10%. It is believed that by introducing SiO_2 nanoparticles, they acted as a surface modifying molecules that optimized the evaporation rate at optimum room temperature within shorter period of time, forming more graded pore sub-layer structure with higher porosity which resulted in an enhanced removal of residual solvent and higher permeation rate through matrices that affected the final behavior of the constructed biosensor [38, 40, 41].

CONCLUSIONS

The paper demonstrates enhanced protocol for construction of hybrid matrices based on sol-gel technology; with optimized addition of prepared SiO_2 particles of precise size in the nano-range at an addition rate of 3% W/W. Immobilized HRP was used as biocatalyst for detection of phenol in presence of hydrogen peroxide. Spectrophotometric analysis was carried out in phosphate buffer (pH 6.0) in the presence of hydrogen peroxide. Results showed that immobilized HRP responds rapidly to changes in substrate addition while having a wide range of detection in our experiments from 0.1 mM till 0.002 M. The resulted signals obtained were proportional to the substrate concentration in samples. Different parameters like pH and temperature were discussed. The stability of immobilized HRP was also demonstrated, about 80% of the initial enzyme activity was retained after 10 successive cycles of application and about 85% of the initial activity 60 days after enzyme immobilization compared to the free HRP that retained about 10–15% after this period. Relative activity for TMOS/CAB/PAMAM (A) and TMOS/CAB/PAMAM (B) were 94% and 90% respectively. The constructed system based on the optimized co-immobilization of HRP demonstrated enhanced operational potential towards further construction of immunosensor.

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ДИЗАЙН НА ОПТИЧНИ БИО-СЕНЗОРИ ЗА ОТКРИВАНЕ НА ФАРМАЦЕВТИЧНИ ПРОДУКТИ

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(Резюме)

„Зол-гел“ синтезът бързо се превърна в завладяваща нова област на изследвания за науката за материалите. Използването на органични молекули в процеса на образуване на гел, може да повлияе на размерите на порите, което представлява друг начин за увеличаване на активността на мобилизирания ензим.

Целта на тази работа е дизайнът на оптични био-сензори за откриване на фармацевтични продукти. Чрез зол-гел методи синтезирахме хибридни матрици, съдържащи силициев диоксид, целулозни производни и поли (амидо/амин) дендримери (PAMAM), като перспективен носители за ковалентна имобилизация. Пероксидазата от *Armoracia rusticana* (HRP) бе използвана като моделен ензим. Условията, които бяха оптимизирани и за които бе определен максимален ефект, са: кинетичните параметри, pH и температурата. Получените био-сензори бяха използвани за детекция на резорцин, pirogallol, епинефрин и т.н. Такива биосензори имат потенциално приложение в медицинската, фармацевтичната, хранителната сфера и за мониторинг на околната среда.