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DESIGN OF OPTICAL BIOSENSOR BASED ON ENZYME IMMOBILIZATION FOR DETERMINATION OF OXIDIZABLE DRUGS

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Abstract: With the increased demand to monitor samples contaminated with small amounts of drugs and toxic compounds, our work aimed at constructing optical biosensor based on horseradish peroxidase (HRP) for quantitative and qualitative detection of oxidizable drugs and toxic compounds in the presence of hydrogen peroxide. HRP was covalently immobilized onto new hybrid membranes synthesized by sol-gel technology. Different types of hybrid membranes were synthesized based on silica precursors, cellulose acetate butyrate (CAB), and PAMAM dendrimers. Horseradish peroxidase was used as a model enzyme. Conditions were optimized including kinetic parameters, pH, and temperature optimums. Results showed that the highest enzyme immobilization and catalytic parameters were achieved by TMOS/CAB/PAMAM based matrices, where relative activity recorded was 90 and 92% for phenol and resorcinol detection respectively, compared to the free HRP, while relative activity of 74% and 85% was measured in the case of CAB/PAMAM based matrices.

The applied system demonstrated enhanced operational potential towards designing biosensor for medical, food industry as well as environmental monitoring purposes.

INTRODUCTION

Developing biosensors, especially optical ones encountered an increased interest over the last decade as a potential successful tool compared to the traditional expensive and complicated techniques; being of high specificity,

efficiency, direct use, reliability and cost effectiveness (Ajeet et al, 2013; Banuls et al, 2013).

The general concept of biosensor is a self-contained analytical device that associates a biological sensing element and a transducer to respond selectively and reversibly to different concentrations of chemical species in monitored samples (Coulet, 1991).

Enzymes are used as biocatalysts in different applications which expanded into new fields like developing biosensors (biological sensing element).

Enhancing enzyme stability can enhance much more their practicality, through reducing their required amounts, prolonging lifetime, or increasing the potential of enzyme reuse, while maintaining a good signal of biosensors (Jungbae et al, 2006).

For developing biosensors, the sol - gel method showed advantages like being versatile, simple and reproducible technique to produce materials with enhanced surface properties, porosity and transparency (Lukowiak et al, 2009). Optical biosensors based on incorporation of biological entities like enzymes within new matrices as the sensing agents are gaining considerable attention. This coupling enables detection of a wide range of compounds from food (Ozsoz and Wang, 1991), pharmaceutical (Deshpande et al., 1990) and environmental activities (Erdem et al., 1998; Ozsoz et al., 1996) and industries. (K›l›nc et al., 1998; Wang and Lin, 1989).

For enzyme incorporation, immobilization approaches like adsorption, covalent attachment, confinement or entrapment onto or within the produced solid structures have to be followed. These immobilization techniques help overcoming enzyme limitations while enhancing their properties (Abdullah et al., 2006) and enzyme stability (Jungbae et al., 2006; Lyubov et al., 2013; Yangyang et al., 2009).

Previous research work reported using peroxidases to catalyze oxygenation of different organic and inorganic substrates in the presence of hydrogen peroxide (Sánchez et al., 1991; Wollenberger et al., 1991). Horseradish peroxidase (HRP) as the most commonly used peroxidase was reported to be used for analytical purposes (Azevedo et al., 2003; Buddhalee et al., 2013).

Alone, HRP, or its conjugates thereof, are of little value, but it must be made visible with a substrate that when oxidized by HRP in presence of hydrogen peroxide, yields a characteristic change that is detectable by spectrophotometric methods.

Signals formed by using peroxidases described in literature could be based on colorimetry (Lyubov et al., 2013), chemiluminescence, fluorescence (Ryan et al., 1994) and amperometric measurements (Arzum et al., 2000).

Covalent immobilization of enzyme onto solid matrices involves immobilization of the enzyme onto the surface of prefabricated matrices and using the resulting conjugates for the sensing process. Using matrices for covalent

immobilization, helps enhancing recovery of immobilized enzymes, which could optimizes operational costs while keeping the enzyme activity preserved (Xianqiao et al., 2004).

Within this study, a system based on HRP covalent immobilization was developed, monitoring of compounds and drugs with oxidizable groups (OxG) like phenol and resorcinol (1,3-Dihydroxybenzene) in the presence of H_2O_2 was done. The biosensor response to the drugs and compounds was monitored. The other parameters like pH, reproducibility, change in H_2O_2 percentage and stability were examined.

MATERIALS AND METHODS

Reagents

Peroxidase isolated from horseradish (E.C. 1.11.1.7) purchased from Sigma–Aldrich; trimethoxysilane (TMOS) from Merck; cellulose acetate butyrate (CAB), PAMAM dendrimers (Polyamidoamine) second generation from Sigma–Aldrich; phenol, resorcinol, 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

Two categories of hybrid membranes were constructed by the sol-gel method with the participation of silica precursor trimethoxysilane (TMOS) along with cellulose acetate butyrate (CAB) as the organic component, and PAMAM dendrimers as a source of groups for covalent immobilization of HRP according to the procedure adopted by our team (Yaneva et al., 2007) and as illustrated in figure 1.

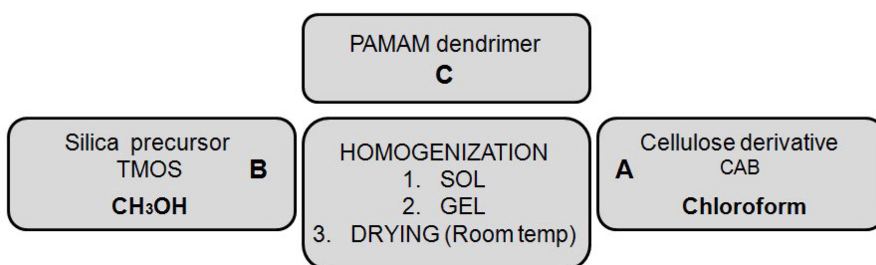

Figure 1. Illustration of sol-gel synthesis of hybrid membranes.

Category 1 (without silica):

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

Category 2 (With TMOS as silica precursor):

Solution A and B were prepared. Solution A was prepared as in category 1, solution B was prepared by dispersing 1ml TMOS in 6ml methanol while using concentrated HCl as a catalyst, stirring the mixture at room temperature for 90 minutes. Then the two solutions were combined, stirred well and dried as in category 1.

Oxidation of carbohydrate residues of Horseradish peroxidase (HRP)

Oxidation of carbohydrate residues of HRP glycoprotein structure to prepare for immobilization was done according to Zaborsky and Ogletree's protocol (Zaborsky and Ogletree, 1974). The oxidized HRP was dialyzed using dialysis membrane obtained from Serva, Germany; by submerging for around 24 hours in 50 mmol/L acetate buffer with pH 5.6. The conditions utilized for oxidation of carbohydrate residues of HRP as well as that used for immobilization step were selected to maximally preserve HRP activity, while achieving high immobilization efficiency (Trevan, 1980). These conditions include the amount of periodic acid, pH, temperature and HRP concentration during immobilization.

Covalent immobilization of peroxidase onto hybrid membranes

Immobilization process to the matrices was achieved through Zaborsky and Ogletree approach, where enzyme immobilization onto activated support is done via covalent linkage between amide groups flanking onto the fabricated matrices to the carbohydrate residues existing within the enzyme structure (Zaborsky and Ogletree, 1974). 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. This procedure is considered a safe way where the immobilization does not affect the enzyme molecule conformation and active sites as binding always takes place far from those active centers.

Determination of enzyme activity and content of protein

The activity of HRP was investigated spectrophotometrically by measuring the increase in absorbance (λ=510 nm) for the solution of oxidized 4-APP (4-aminoantypyrin), as it is converted to the reduced form by HRP. HRP specific activity is expressed in terms of pyrogallol units. One pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C. The assay was done in phosphate buffer (PBS, 100 mM, pH 6.5) containing 4-APP (1.4 ml, 2.5 mM), H_2O_2 (1.4 ml, 1.7 mM), at 25°C. Total protein content was determined using modified Lowry method, with bovine serum albumin as standard (Lowry et al., 1951).

V_{max} and KM determination

Using Lineweaver-Burk plots for characterization of the immobilized HRP, the apparent Michaelis constants and maximum velocity of the reaction were calculated, and kinetic parameters of immobilized HRP were compared with parameters of free HRP.

pH and temperature optimum

The optimum operational pH for immobilized HRP was determined in 100 mM phosphate buffer in the pH range 4.0- 8.0 using 100 µM solution of hydrogen peroxide. Temperature optimum was determined in the range from 20°C to 60°C.

RESULTS AND DISCUSSION

The two categories of constructed hybrid membranes exhibited good uniformity, flexibility, transparency and surface properties, which is desirable criteria for sensing applications and enzyme immobilization, with the included biocompatible cellulose derivatives and silica. On the other hand, membranes exhibit inertness towards the enzyme, in addition to being of low preparation cost, simply prepared through sol-gel process (Brodelius et al., 1987; Trevan, 1980)

It was reported that the used inorganic silica precursors used to integrate within the cellulose matrix forming hydrogen bonding (connecting both organic and inorganic phases) while decreasing the internal pressure gradients during drying step (Xie et al., 2009). Using PAMAMs as being a polymeric material might affect the inorganic condensation-polymerization process and that of the sol-gel material (Gupta and Chaudhury, 2007)

Catalytic properties of free and immobilized HRP

Table 1 presents Results for catalytic properties of immobilized HRP onto hybrid membranes synthesized with CAB/PAMAM and TMOS/CAB/PAMAM.

Table 1. Catalytic properties of free and immobilized HRP.

<table>
<tr><th>Sample</th><th>Abs. dry wt. [mg/g]</th><th>Specific activity [U/mg]</th><th>Relative activity [%]</th><th>pH optimum</th><th>Temp. Optimum [°C]</th></tr>
<tr><td>Free HRP (Phenol)</td><td>-</td><td>78.9</td><td>-</td><td rowspan="2">6.0</td><td rowspan="2">25</td></tr>
<tr><td>Free HRP (Resorcinol)</td><td>-</td><td>49.2</td><td>-</td></tr>
<tr><td>CAB/PAMAM (Phenol)</td><td rowspan="2">1.7</td><td>59</td><td>74</td><td rowspan="2">6.5</td><td rowspan="2">35</td></tr>
<tr><td>CAB/PAMAM (Resorcinol)</td><td>41.9</td><td>85</td></tr>
<tr><td>TMOS/CAB/PAMAM (phenol)</td><td rowspan="2">2.1</td><td>71.3</td><td>90</td><td rowspan="2">6.0</td><td rowspan="2">40</td></tr>
<tr><td>TMOS/CAB/PAMAM (Resorcinol)</td><td>45.3</td><td>92</td></tr>
</table>

By comparing HRP catalytic properties, TMOS/CAB/PAMAM showed better results, with the highest relative activity recording 90% and 92%, while CAB/PAMAM relative activity was 74% and 85% with phenol and resorcinol respectively. On the other hand, under pH 7.0, TMOS/CAB/PAMAM were of the higher levels of bounded enzyme followed by CAB/PAMAM recording 2.1 mg and 1.7 mg bounded protein per gram absolute dry weight respectively. The reason for this might be that using CAB with its high surface irregularities and

porosity of the constructed membranes, has provided more surface area for higher quantity of enzyme immobilization and substrate penetration into the membrane and the products out by decreasing substrate diffusion limitation, thus having CAB membranes with more open structure at the end (Bayramoglu et al., 2013; Manuela et al., 2008).

Using silica precursors in the gel formation process has affected resulted pores, while offering greater protection from the environment, explaining the resulted increase in enzymatic activity within samples (Beatriz et al., 2006).

Results presented in table 2 from kinetic parameters of immobilized HRP were compared with parameters of free HRP. Km values obtained were found varying between 0.76×10^{-3} mM and 2.62×10^{-3} mM. Kinetic parameters results corresponded with the results reported by Mohamed and co-authors (Mohamed et al., 2008).

As shown in figure 2 (a and b), immobilized and free HRP exhibited response to the reduction of phenol and resorcinol concentration with a recorded range from 0.09mM/L to 1.8 mM/L for phenol and from 0.1mM/L to 2.2mM/L for resorcinol.

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

Sample	K_m [µM]	V_{max} [µM/min]	R^2
Free HRP (phenol)	2.62	37.03	0.989
Free HRP (resorcinol)	0.91	14.5	0.980
CAB/PAMAM (phenol)	1.03	15.62	0.997
CAB/PAMAM (resorcinol)	1.38	9.09	0.989
TMOS/CAB/PAMAM (phenol)	2.46	33.33	0.993
TMOS/CAB/PAMAM (resorcinol)	0.76	7.35	0.984

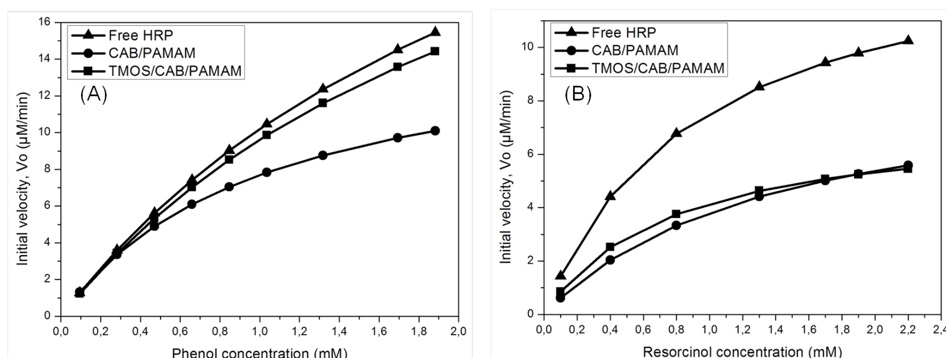

Figure 2. Dependence of the rate of the enzyme catalyzed reaction on the substrate concentration – phenol and resorcinol.

Figure 3 shows the dependence of the steady state of immobilized HRP on the concentrations of different oxidizable compounds: phenol and resorcinol. For phenol, the increase in response was linear up to 1.1 mM/L for free HRP, 1.8 mM/L for CAB/PAMAM and 1.3 mM/L for TMOS/CAB/PAMAM. While for resorcinol, a wider range of linearity was observed for free HRP and CAB/PAMAM to 2.2 mM/L, while TMOS/CAB/PAMAM recorded a linear response up to 1.7 mM/L. Results coincides with Arzum et al (Arzum et al., 2000).

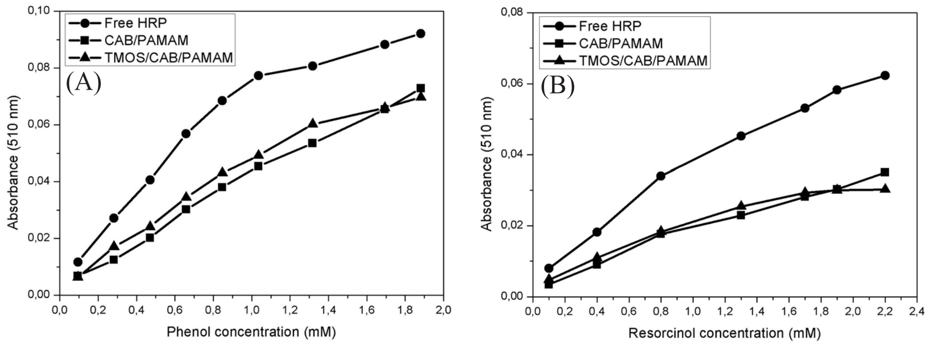

Figure 3. Dependence of the absorbance on concentration of phenol and resorcinol.

As shown in fig. 4 (A) and as a function of temperature, results showed a more general stability of Immobilized 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 TMOS/CAB/PAMAM, where matrix protection for HRP found to be affecting temperature optimums and enzyme stability, These observations corresponds with work done by Yotova et al (Lyubov et al., 2013).

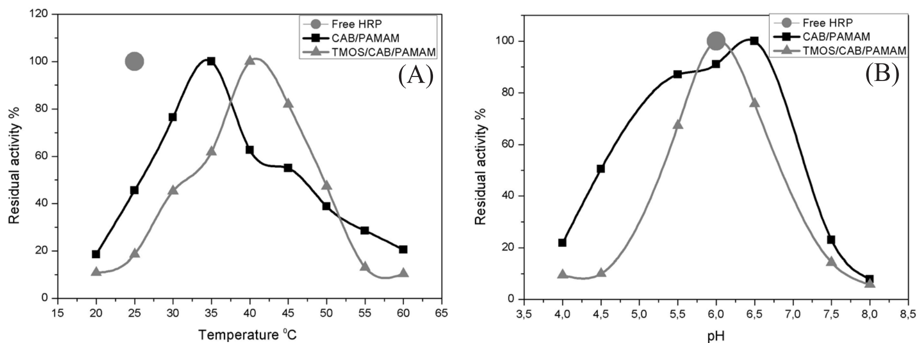

Figure 4. (a) Temperature effect on residual activity of immobilized HRP at pH 6.0.
(b) pH effect on residual activity of HRP at room temperature.

Figure 4 (B) shows the optimum operational pH, 100% residual activity remained the same for TMOS/CAB/PAMAM immobilized HRP compared with free HRP samples around pH 6.0, while a shift was observed to pH 6.5 that was

recorded by CAB/PAMAM. In general immobilized HRP curves showed relatively high residual activity over a wider pH range, which goes with previously reported results (Yin et al., 2009).

CONCLUSIONS

This paper demonstrates the advantages of using immobilized HRP as biocatalyst in in vitro detection of compounds like phenol and resorcinol in presence of hydrogen peroxide. Spectrophotometric analysis was carried out in phosphate buffer (pH 6) in the presence of hydrogen peroxide, while adding compounds with an oxidizable group (OxG); e.g. -OH, -NH, -SH. The results showed that immobilized HRP responds rapidly to changes in substrate addition, while having a wide range of detection in our experiments from 0.1mM to 0.002M. 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. The results showed that the relative activity recorded by CAB/PAMAM and TMOS/CAB/PAMAM was 74% and 90% for phenol detection and 85% and 92% for resorcinol respectively. The constructed system based on the optimized co-immobilization of HRP demonstrated enhanced operational potential towards further construction of immunosensors.

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