Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Online publication date: 19 March 2003

To cite this Article Amounas, Mohamed , Innocent, Christophe , Cosnier, Serge and Seta, Patrick(2003) 'Dismutation of Hydrogen Peroxide from Water Medium by Catalytic Reactive Membrane Immobilizing Peroxidase and Catalase by Molecular Recognition Process', Separation Science and Technology, 38: 6, 1291 — 1306
To link to this Article: DOI: 10.1081/SS-120018810
URL: http://dx.doi.org/10.1081/SS-120018810

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SEPARATION SCIENCE AND TECHNOLOGY
Vol. 38, No. 6, pp. 1291–1306, 2003

Dismutation of Hydrogen Peroxide from Water Medium by Catalytic Reactive Membrane Immobilizing Peroxidase and Catalase by Molecular Recognition Process

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ABSTRACT

Electropolymerized polypyrrole–biotin on carbon felt has been processed as filtration membranes immobilizing biotinylated enzymes (peroxidase and catalase) by the avidin–biotin recognition process. These membranes are shown to be efficient systems for hydrogen peroxide dismutation because the concentration of active enzyme in the membrane is high in comparison with that obtained by current techniques for enzyme immobilization. A Michaelis–Menten kinetic analysis type is proposed, which demonstrates that even if the rate of

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DOI: 10.1081/SS-120018810 0149-6395 (Print); 1520-5754 (Online)

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the enzymatic process is decreased in the membrane environment, the corresponding decrease of the rate parameters is no more than a factor of four compared with values in solution. The long-term stability and reactivity of the enzymatic membrane were also shown to be significantly improved compared with other types of routes for enzyme immobilization.

Key Words: Reactive membranes; Immobilized enzymes; Molecular recognition; Kinetics.

1. INTRODUCTION

The concept of the membrane reactor has been increasingly researched in recent years because it has been postulated that the membrane core of a filtration membrane could itself be a reactive phase. Thus, the membrane would bring in addition the ability to chemically transform compounds that are solubilized in the permeate. This principle is equally valid for liquid as well as for gas transformation.[1] This type of chemical transformation is very efficient because the process is a catalytic one. The immobilization of enzymes that are efficient catalysts with high turnover for the transformation of organic and inorganic water soluble molecules has been the subject of several studies.[2] The interest in such systems is increasing especially in the domain of water and also in the bio or food industries.

In a previous article we described the immobilization of biotinylated glucose oxidase and peroxidase by a specific and irreversible biotin–avidin molecular recognition process.[3] In the present article, we generalized this process of enzyme immobilization to another enzyme (i.e., catalase), which has been biotinylated prior to membrane immobilization because catalase is not a commercially available biotinylated enzyme. We have measured the kinetic parameters of the enzymatic reaction (hydrogen peroxide dismutation) in the membrane system. A comparison of the deduced rate parameters with those of the free respective enzymes has shown a slight decrease in the reactivity of the enzymes immobilized on the polypyrrole membrane compared to that for the free enzymes, but far from the loss of reactivity generally observed until now with enzymes immobilized by using more classical routes such as chemical bonding.[4] Moreover, the long-term stability of the membrane in terms of reactivity also appeared to increase substantially.

2. EXPERIMENTAL SECTION

2.1. Reagents

Avidin (chromatographically purified), biotin-labeled peroxidase (B-POD), (E.C. 1.11.1.7., 240 U mg^{-1}), and catalase (E.C. 1.11.1.6, 2225 U mg^{-1}) were purchased from Sigma. The pyrrole monomer **1** (12 pyrrol-1-yl dodecyl biotin) was synthesized as previously reported.[5] All other reagents used were of analytical grade and purchased from Fluka.

2.2. Apparatus

Electrochemical experiments were performed with a Tacussel model PRT 30-0.1 potentiostat equipped with a Tacussel GSTP3 signal generator and a Kipp & Zonen BD91 recorder. Electrochemical polymerization of the pyrrole–biotin monomers were carried out in a classical three-electrode cell commonly used in cyclic voltammetry experiments. An Ag/AgCl electrode (Tacussel model 201) was used as a reference electrode and a platinum wire as counter electrode. The working electrode was carbon felt ($1.5 \times 0.5 \times 0.5$ cm).

Spectrophotometric measurements were performed by using a Uvikon 940 spectrophotometer (Kontron Instruments). Hydrogen peroxide dismutation was achieved with a flow of 30 cm^3 hydrogen peroxide 10^{-2} M solution across a membrane held in a column as shown in Fig. 1 and described in Sec. 2.5. Absorbance measurements were carried out in spectrophotometric ($1 \times 1 \times 4$ cm) circulation cells (Hellma France), connected to the column exit.

2.3. Biotinylation of Catalase

A biotinylated catalase was prepared by adding a fixed amount (2 mg ml^{-1}) of catalase solution (phosphate buffer 100 mM pH 6.0) to 100 times molar excess of biotinamidocaproate N-hydroxysuccinimide ester. The biotinylated reaction was carried out in a vial under constant stirring at room temperature during 3 hr. At this time, 10 mg of glycine was added to react with the unused biotin. Removal of all small molecular weight reactants and products was achieved by chromatography through a Sephadex G 25 column from Sigma (St. Louis, MO) (10000 MW/cut off).

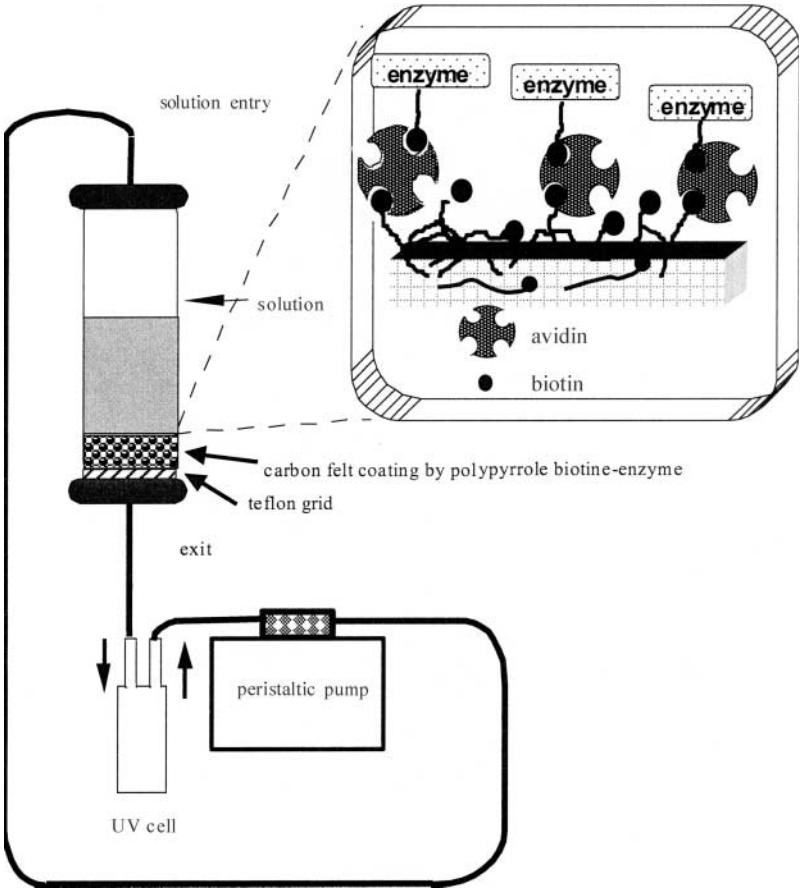

Figure 1. Schematic diagram of the enzymatic membrane device. Glass column dimensions: diameter: 1 cm; height: 10 cm. The inset represents the enzyme linkage of the biotinylated enzyme to the electropolymerized polypyrrole–biotin by the avidin–biotin molecular recognition process.

2.4. Enzymatic Membrane Preparation

The enzymatic membrane was prepared in a three-electrodes cell as follows: monomer **1** (2 mM) in 0.1 M $NaClO_4$ acetonitrile was electropolymerized on a $1.5 \times 0.5 \times 0.5$ cm carbon felt from Carbone Industrie-France).

The specific surface per volume unit of about $42\,cm^2$ per cm^3 has been estimated as in Ref.[6] by recording the electrochemical signal of reduction of an electroactive compound such as ferricyanide ion. The porosity of the felt used is about 95%.[7]

The polypyrrole–biotin-modified support was rinsed with distilled water and immersed in 2 mL of avidin aqueous solution ($1\,mg\,mL^{-1}$) during 1 hr. The resulting porous carbon membrane was washed with distilled water and immersed in 2 mL of biotinylated enzyme solution ($2\,mg\,mL^{-1}$) during 1 hr and then washed again with distilled water.

2.5. Membrane Device

The modified carbon felt was tightly packed (0.5 cm in thickness) into a 1 cm in diameter 10-cm-height glass column for chromatography. The column was filled with the studied solution at the top of the column, and the solution flowing through the membrane was analyzed at the exit (Fig. 1). The solution was circulated by pumping with a peristaltic pump and maintained at 20°C. Because of the high porosity of the felt, no strong limitation of the liquid flow occurred in the column during the filtration process.

2.6. Hydrogen Peroxide Measurements

The following analytical methods[8] (peroxisase activity determination from Sigma) were used to detect hydrogen peroxide concentration changes:

1. Reduction of H_2O_2 catalyzed by peroxidase in the presence of pyrrogallol: A mixture of pyrogallol (at a concentration not lower than the hydrogen peroxide one) and peroxidase was added to the hydrogen peroxide aqueous solution. The measured absorbance change at 420 nm corresponding to purpurogallin formation ($\varepsilon = 248\,M^{-1}\,cm^{-1}$) is directly related to the H_2O_2 concentration decrease according to the Eqs. (1) and (2):

$$H_2O_2 + POD_{red} \rightarrow POD_{ox} + H_2O \quad (1)$$

$$POD_{ox} + \text{pyrrogallol} \rightarrow \text{purpurogallin} + POD_{red} \quad (2)$$

2. Cooxidation of phenol and antipyrine catalyzed by peroxidase: A mixture of phenol, 4-amino-antipyrine (at concentrations not lower than half the concentration of hydrogen peroxide) and peroxidase was added to the hydrogen peroxide aqueous solution. The change of absorbance was recorded

at 505 nm ($\varepsilon = 6.4 \times 10^3\ M^{-1}\ cm^{-1}$), which corresponds to the wavelength maximum of the absorption band of the quinone-imine produced by the cooxidation of phenol and antipyrine catalyzed by the peroxidase.

3. For high concentrations of hydrogen peroxide ($> 10^{-2}$ M), a direct spectroscopic measurement was used. The optical density of the solution was recorded at 240 nm, and the resulting concentration was calculated by using a value of 39.4 $mol^{-1}\ cm^{-1}$[9] for the molar extinction coefficient of H_2O_2.

3. RESULTS AND DISCUSSION

3.1. Poly(pyrrole–biotin) Film Characterization

The structure of the film was assessed by scanning electron microscopy. The polymerization of the pyrrole–biotin monomer was carried out on a carbon felt. Figure 2 shows photographs of polymer deposition on the carbon surface. The latter illustrates that the electrochemical addressing of polymer films allows the spatially controlled functionalization of surfaces whatever their shape and size are. We recently demonstrated by gravimetric measurements that the immobilization of avidin on biotinylated polypyrrole films via bio-affinity interactions provides a compact avidin monolayer.[10] Figure 2C shows this specific binding event of avidin onto polymerized biotin leading to a smoothed polypyrrole–air interface.

3.2. Peroxidase Immobilization

3.2.1. Amount of Immobilized Enzyme

The amount of POD immobilized on the carbon felt after electropolymerization was determined by enzymatic activity measurements. The enzyme solutions were analyzed before and after contact with the carbon tissue, and the amount of enzyme actually grafted in the polymer matrix was calculated in the same way as previously described in the experimental section. The result demonstrates that 1.6 mg of enzyme was anchored in the polymeric matrix, corresponding to 83% of the initial amount of enzyme (2 mg) put in contact with the carbon felt modified by the polymer. The amount of fixed active enzyme in the membrane is in the range of 4.1 $mg\ cm^{-3}$, which corresponds to approximately 0.1 $mg\ cm^{-2}$ of the effective surface. Taking into account that the theoretical maximum surface coverage for a closely packed avidin monolayer corresponds to $3.3–5 \times 10^{12}$ molecules cm^{-2}[4]; the maximum

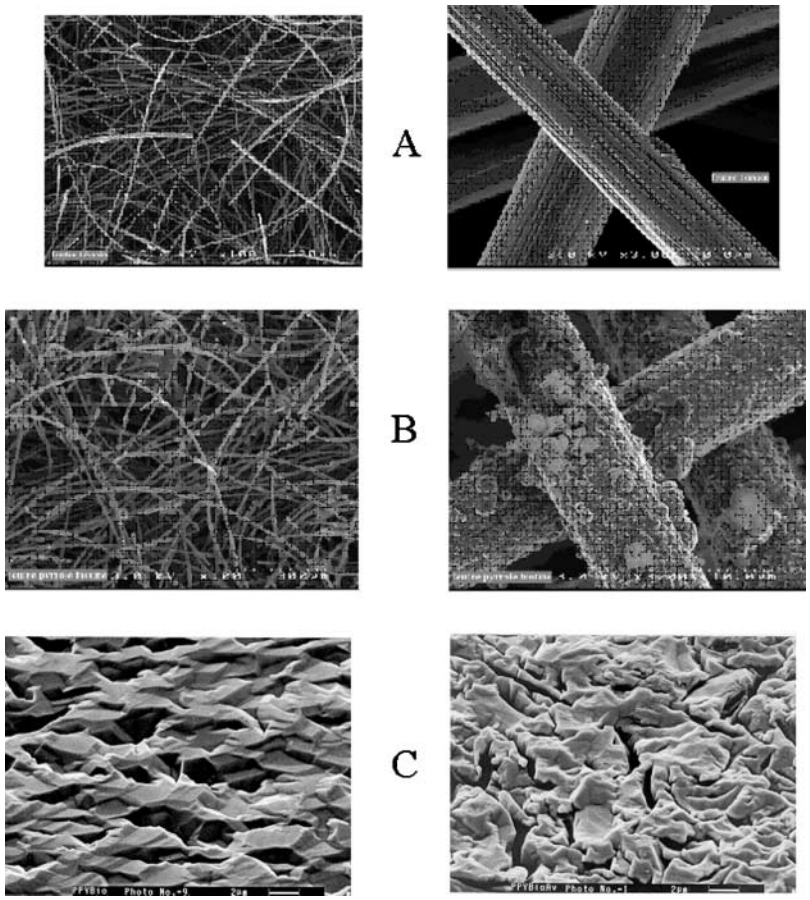

Figure 2. Micrographs of carbon felt before (A) and after modification by electropolymerization of pyrrole–biotin monomer (B) and deposited on platinium surface (C) before (*left*) and after (*right*) complexation with avidin.

amount of anchored POD should be $0.22-0.33\,mg\,cm^{-2}$. The specific anchoring of POD by avidin–biotin bridges thus constitutes an efficient approach for the functionalization of the whole structure of a carbon felt. Specific recognition between avidin and biotinylated enzyme allows a strong increase of enzyme grafting compared with other matrices.[11] Thus, the complexation between biotin and avidin appears to be limited by steric interaction, with the smaller enzyme size, the more efficient immobilization.

The same result has been reported for enzyme immobilization in the case of polypyrrole derivatives for biosensor elaboration.[12]

3.2.2. Membrane Working

Equimolecular aqueous solution of H_2O_2 and pyrrogallol (5×10^{-3} M) passed through the POD membrane and were analyzed by absorption spectroscopy. In the course of the transfer across the membrane, H_2O_2 is reduced by the immobilized POD, which in turn oxidizes pyrrogallol into purpurogallin [see Eqs. (1) and (2)]. The amount of purpurogallin detected corresponds to the quantity of H_2O_2 consumed by the enzymatic reaction. The chemical yield of the membrane process corresponds to the ratio between the number of purpurogallin molecules produced and the number of H_2O_2 molecules introduced in the column. A value of 0.8 was obtained for this ratio. This value, although satisfying by itself, can be improved by a recirculation of the feed solution through the enzymatic membrane.

After a recirculation time of 30 min (which corresponds to six passages of the feed solution volume through the membrane), all of the initial H_2O_2 was oxidized by the enzymatic catalytic membrane.

Figure 3 shows the rate of consumption of hydrogen peroxide (initial concentration 5×10^{-3} M) vs time whereby the exit solution is continuously

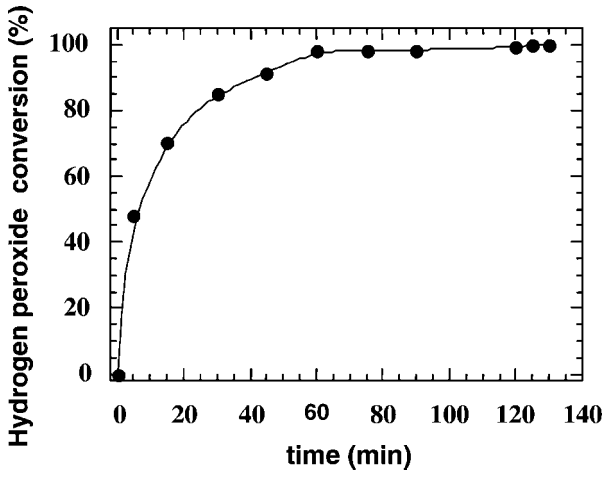

Figure 3. Conversion rate vs. time of hydrogen peroxide solution (10^{-3} M) with peroxidase membrane (membrane thickness: 1 cm; flow rate: 1 mL min^{-1}).

injected into the column, thus allowing a constant flow through the enzymatic membrane. The remarkable efficiency of this hydrogen peroxide degradation process is explained by the peculiar molecular interaction process used for the enzyme attachment in the immobilization procedure. In this way, the enzyme was neither altered nor denaturated owing to avidin–biotin complexation, and the catalytic activity was kept up in the membrane. The measurement of the specific activity of the free POD in solution related to pyrrogallol transformation into purpurogallin indicates 200 U mg^{-1} of enzyme (value not very far from that notified by the seller, 240 U mg^{-1}). The membrane enzyme activity determined in the same experimental conditions was 117 U mg^{-1}. This value corresponds to 58% of free enzyme activity. Thus, the remaining activity of the immobilized enzyme is markedly higher than those previously reported with other immobilization methods based on electrogenerated polymer films (only 4% of free peroxidase activity[4] and 9% of free polyphenol oxidase activity[10] are kept up in the polymer matrix). This clearly illustrates the advantages offered by a soft attachment of the enzyme molecules which preserves their conformational flexibility and hence their activity.

3.2.3. Dependence on pH

The optimum pH range for reactivity of the catalytic membrane was investigated with hydrogen peroxide (5×10^{-3} M) as substrate and a circulation time of 15 min (flow rate: 2 mL min^{-1}). In the pH range of 4.7–8.3, the enzyme membrane shows a typical bell-shaped response. The maximum efficiency is obtained for pH 6.8. (Fig. 4). This value is in good agreement with that determined with free POD in solution.[13] The kinetic experiments presented below were performed out at pH 6.8, which corresponds to the pH of maximum efficiency for the immobilized enzymes.

3.3. Kinetic Parameters

Kinetic analysis was carried out by substrate concentration recording of the solution at the column exit.

According to the investigations previously reported,[14] the immobilized enzyme reactivity in the carbon felt was estimated by considering the residence time of the substrate solution in the membrane. The mean residence time t_r of solute in the porous material can be calculated by the following

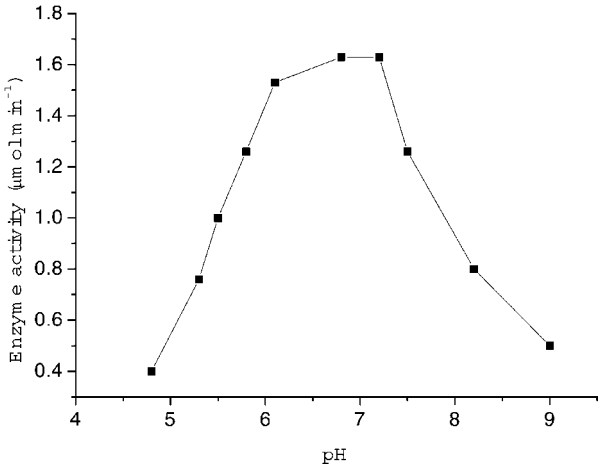

Figure 4. Effect of pH on the enzyme activity of the membrane.

equation:

$$t_r = L/J$$

where L is membrane thickness and J is volume flux of solution. J is equal to the ratio of flow to membrane surface.

The reaction rate is calculated as

$$V = \Delta C/t_r$$

where ΔC is the concentration decrease of hydrogen peroxide in the flowing solution.

3.3.1. Peroxidase Membrane Case

Figure 5A depicts the relationship between the reaction rate and substrate concentration in the case of a POD membrane. The rate follows a classical enzymatic kinetic curve. The first step corresponds to a linear part (weak concentration of substrate). For high substrate concentrations, a reaction rate saturation is observed, and the maximal value obtained is due to saturation of active enzyme sites.

Figure 5B shows the corresponding Lineweaver-Burk plot for the immobilized POD membrane. From the obtained slope (K_M/V_M) and intercept

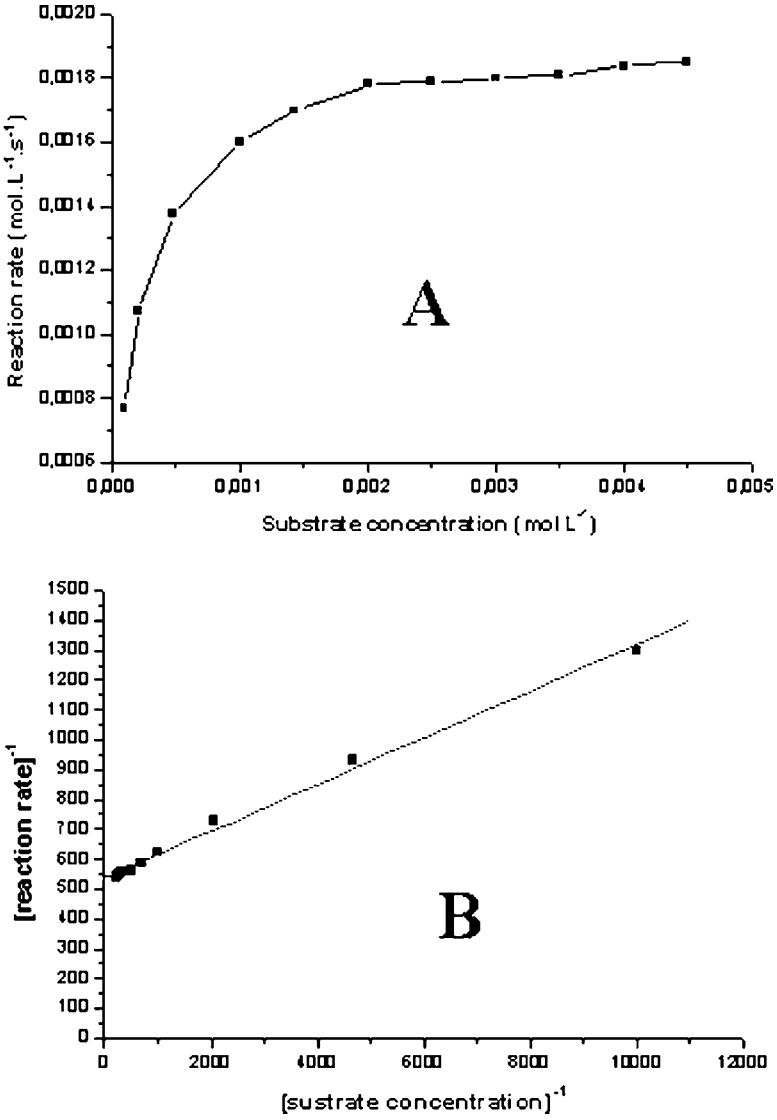

Figure 5. Reaction rate of hydrogen peroxide dismutation with POD–polypyrrole membrane (A) and Lineweaver-Burk plot of the POD–polypyrrole membrane (B); $t_r = 42$ s; membrane thickness: 1 cm; flow rate: 1 mL min^{-1}.

($1/V_M$) for the linear relationship, both K_M and V_M are estimated in the range of 1.45×10^{-4} M and 1.8×10^{-3} M s^{-1}, respectively.

3.3.2. Catalase Membrane Case

To use the enzyme membrane for hydrogen peroxide dismutation, immobilization of catalase has been achieved.

A simplified scheme for the reaction mechanism of catalase may be described by the following two equations[15,16]

$$\text{cat(OH)}_4 + \text{H}_2\text{O}_2 \leftrightarrow \text{cat(OH)}_3\text{OOH} + \text{H}_2\text{O}$$

$$\text{cat(OH)}_3\text{OOH} + \text{H}_2\text{O}_2 \rightarrow \text{cat(OH)}_4 + \text{O}_2 + \text{H}_2\text{O}$$

where cat(OH)_4 is native catalase and cat(OOH)_3 the oxidized enzyme complex.

The kinetic study was carried out both with the peroxidase and the catalase membrane to understand the effect of immobilization by avidin–biotin technology.

In some cases, the treatment of the kinetics results by a classical Lineweaver-Burk plot gives a negative value for the extrapolated reaction rate. This finding suggests that the maximum reaction rate of enzymatic reaction is negative, which does not make any sense. For instance, for cases in which zero or negative y-intercepts are obtained, the K_M is much larger than the total experimentally accessible substrate concentration and the simplified Michaelis–Menten model does not apply. The same phenomenon was previously observed with another enzyme such as lipase.[17] It is the reason why in Table 1 we preferred to use the catalytic constant (k) to compare the activity of the different enzymes.

Table 1. Kinetic parameters: Michaelis constants (V_M and K_M) for catalase and peroxidase.

	k*[E]	k ($s^{-1}\,mg^{-1}$)	K_M ($mol\,L^{-1}$)	V_M ($mol\,L^{-1}s^{-1}$)
Free catalase	10^{-2}	0.125	3.26×10^{-2}	
Biotinylated catalase	9×10^{-4}	0.011	9.5×10^{-3}	0.11
Immobilized catalase	5×10^{-2}	0.031	7	3×10^{-2}
Immobilized peroxidase			1.45×10^{-4}	1.8×10^{-3}

Table 2. Reaction rate and activity units of enzymes.

	Reaction rate 0.1 M H_2O_2 ($M\,s^{-1}$)	V ($M\,s^{-1}\,mg^{-1}$)	Activity units per mg of solid enzyme ($U\,mg^{-1}$)
Free catalase	10^{-3}	0.0125	2250
Immobilized biotinylated catalase	5×10^{-3}	0.0031	562
Immobilized HRP	0.28×10^{-3}	0.000175	506[a]

[a] From Ref.[20,21]

The catalytic constant (expressed in $s^{-1}\,mg^{-1}$ normalized to a constant amount of enzyme) for the immobilized catalase is defined as the ratio of the slope of the curve reaction rate vs. substrate concentration. This value is four times lower than that of the free catalase. Such a result is in good agreement with those values obtained for catalase immobilized on cellulose.[18] However, it is interesting to stress the fact that for catalase the biotinylation of the enzyme decreases the rate by a factor of about 10, whereas the immobilization enhances the rate compared with that of the biotinylated enzyme in solution. The K_M value drastically increases when catalase is immobilized by avidin–biotin recognition. This value is not a significant physical parameter because it corresponds to an unrealistic concentration of hydrogen peroxide. At these high concentrations, the catalase should be totally deactivated by the substrate.[19] Enzyme immobilization for instance in polymeric hydrogels[20,21] has been shown to exhibit large K_M value (Table 2).

A strong increase of K_M means that the kinetic parameters are affected by the enzyme immobilization, presumably due to the steric effects, which significantly decrease the catalytic reaction rate.

3.5. Membrane Stability

The enzymatic membrane was stored at 4°C in buffer solution. Assays were performed at different storage times. Figure 6 illustrates membrane stability measured in the case of POD immobilization as the hydrogen peroxide concentration converted to purpurogallin, starting from a 5×10^{-5} M equimolar H_2O_2–pyrrogallol aqueous solution flowing across the membrane. In catalase immobilization, the same operational stability with initial concentration of H_2O_2 2×10^{-2} M was recorded. No significant decrease in enzyme activity was observed after 140 days and 8 hr of cumulative

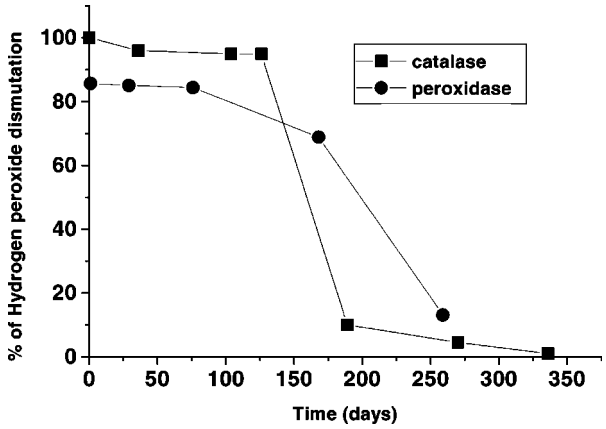

Figure 6. Enzyme activity (in %) of the POD-membrane (●) and catalase membrane (■) after storage at 4°C in buffer solution. Activity were measured with hydrogen peroxide concentration of 10^{-3} M after circulation time of 20 min (flow rate: 1 mL min^{-1}).

dismutation reaction with a flow rate of 2 mL min^{-1}. The enzymatic membrane was remarkably stable in comparison with membranes made by other immobilization methods[4,11] and thus it should be noted that the enzyme was actually irreversibly immobilized.

In catalase immobilization, the stability is also satisfying: a loss of enzyme activity being only observed after 140 days of storage in buffer solution at 4°C.

3.6. Conclusion

In this work, the efficiency of the immobilization method based on avidin–biotin technology linked to an intermediate electropolymerized polymer layer onto carbon fibers for the building of a catalytic filtration membrane has been demonstrated. The enzyme activity of this new type of membrane along with the kinetic parameters have been determined in the case of the dismutation of hydrogen peroxide. These rate parameters are not too much affected by the immobilization process because the loss of reactivity compared with free enzymes was shown to be moderate. Moreover, the long-term stability of the catalytic activity of this type of reactive membrane make them promising candidates for practical application in membrane technology for liquid transformations.

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Received March 2002
Revised August 2002