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Horseradish Peroxidase Immobilized Through Its Carboxylic Groups onto a Polyacrylonitrile Membrane

Comparison of Enzyme Performances with Inorganic Beaded Supports

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Abstract

A hydrophilic polyacrylonitrile (PAN) flat sheet membrane was aminated (8.5 µmol of NH_2/mg of dry support) for covalent binding of horseradish peroxidase (HRP), mediated by the soluble carbodiimide l-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). Silica microbeads derivatized by silanization, to yield an aminated support, and commercial aminated glass microbeads were also coupled to HRP with EDC or activated with glutaraldehyde. The immobilized enzyme activities were determined in a batch enzyme reactor with an external loop, the highest specific immobilized HRP activity being obtained on the glass support (55.8 U/mg of protein). Continuous operational stability studies showed that hydrophilic PAN membrane led to the highest retention of HRP activity after an overall period of 35 h, with a normalized productivity of 59.5 µmol of H_2O_2 reduced/($h \cdot U_{immob\ HRP}$).

Index Entries: Horseradish peroxidase activity; stability; immobilized enzyme productivity; polyacrylonitrile membrane.

Introduction

Peroxidases can be used for rapid (or online) detection methods of several analytes in mammalian body fluids, in pharmaceutical and biotechnological processes, or in commercial food samples. A stable peroxidase is

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often used as the second enzyme in a dual-enzyme analytical method, the main enzyme being the analyte oxidase. The analyte oxidase stoichiometrically produces H_2O_2 which is reduced by the peroxidase. The end point of this second reaction leads to the indirect quantification of the analyte. Especially adequate analytes are all those for which detection based on the principle of amperometric enzyme membrane electrode with H_2O_2 oxidation has been studied (e.g., D-glucose and L-lactate, through glucose oxidase and lactate oxidase, respectively) *(1–3)*. In this enzymatic method, a color precursor together with the H_2O_2 develops a colored product (chromogenic system). Fluorescent precursors can also enzymatically react with H_2O_2 to form a fluorescent product *(4)*, allowing quantification of low levels of H_2O_2. This method was used in environmental control, monitoring H_2O_2 in rainwater *(3)*. Toxic phenolic residues have been treated with peroxidases *(5)*, suggesting that phenolic residues could also be quantified in wastewater through single-enzyme peroxidase sensors/analysis.

In spite of the potential application of peroxidases as analytical tools or process catalysts, a more extensive use has been impaired by the low operational stability of the immobilized enzyme *(5–7)*.

Recently, several investigators have developed methods to increase the stability of horseradish peroxidase (HRP) *(7–9)*. Silica microparticles (~2.5 µm) were used for HRP immobilization through glutaraldehyde coupling. These particles were used for catalysis in organic solvents with increased stability *(7)*. Thin films of SiO_2 (100 nm thick) and of glass (150 µm) with immobilized HRP have been characterized *(8)*. The enzyme coupling was ensured by silanization with 3-aminopropyl-methyl-diethoxysilane followed by a succinylation step and a carbodiimide/*N*-succinimide activation of the support. Alkylamine glass beads (55-nm pore diameter) were also used for the development of an oxalate analysis technique based on a dual-enzyme methodology on HRP immobilization using glutaraldehyde as bifunctional coupling reagent *(9)*.

The main purpose of the present work was to study and characterize an immobilization methodology based on the use of organic membranes as enzyme matrices leading to high levels of HRP activity and long-term operational stability. Different activation procedures were tested on selected solid supports, including a hydrophilic polyacrylonitrile (PAN) microfiltration membrane and controlled pore silica and glass beads.

Materials and Methods

Supplies and Chemicals

UltraFilic® membranes of 0.2-µm nominal pore size (chemically modified PAN of very high hydrophilicity [see www.osmonics.com/products/page918.htm for product information on Osmonics UltraFilic membranes]; type MX-2000; 4° contact angle) was kindly supplied by Membrex (which was acquired by Osmonics, Minnetonka, MN in 1998). Porous silica microparticles ($63 < d_p < 200$ µm; $d_{pore} \sim 500$Å) were from Riedel-de-Hahen,

Germany; and porous aminopropyl glass spherical beads ($74 < d_p < 125$ µm; $d_{pore} \sim 500$Å) were from Sigma, St. Louis, MO.

Lithium aluminum hydride ($LiAlH_4$), 5% 2,4,6-trinitrobenzene-sulfonic acid (TNBS) solution, 3-aminopropyltrimethoxysilane (APTS), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were of analytical grade and also from Sigma. Phenolsulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) were both from Aldrich. Freeze-dried HRP (90% purity) was from Bioenzyme. Bovine serum albumin ([BSA], fraction V) standard solution was from Pierce.

Activation of PAN Membranes

Several 100-mm^2 membrane pieces were cut from a flat sheet and washed by immersion in 15 mL of distilled water for 24 h. After the removal of excess water with an absorbing cloth, the membrane pieces were kept 4 h at 80°C until constant dry weight, weighted (11 ± 0.6 mg dry wt/cm^2), and stored for further utilization. The reported areas are external membrane surface areas rather than total surface areas (not including internal pore surface).

The nitrile functional groups (–CN) available on the membrane surface were reduced to a primary amine *(1)*. Each membrane piece was wetted (removing entrapped air inside the pores) and immersed in 100 m*M* $LiAlH_4$ solution in dried ethyl ether. Reaction occurred in a moisture-free environment (orbital shaker at 25°C for 3 h). Afterward, a washing step was performed in a vacuum filtration funnel with 50 mL of 1 *N* HCl, followed by 50 mL of 1 *N* NaOH and 100 mL of H_2O.

Derivatization of Inorganic Supports

The porous silica was activated with APTS according to the silanization process described by Weetall *(10)*. To each 50 mg of HNO_3-washed supports 1 mL of aqueous 8% (v/v) ATPS (pH 3.0–4.0) was added. After incubation (2 h at 80°C) the aminated supports were washed and dried.

Glutaraldehyde Coupling of HRP

The carbonyl derivative of the alkylaminated inorganic beads was obtained by glutaraldehyde activation *(10,11)* (3% [v/v] glutaraldehyde in 50 m*M* phosphate buffer, pH 7.0). The carbonyl derivative support (50 mg) was incubated at room temperature (22 ± 2°C) for 16–24 h with 1 mL of enzyme solution (15 mg of HRP/mL of the phosphate buffer). The supernatant reaction fluid was removed, and the immobilization supports were washed with 20 mL of the buffer solution. Excess buffer was removed and the immobilized enzyme preparation maintained wet at 4°C before evaluation of its peroxidase activity.

Carbodiimide Coupling of HRP

Aminated matrices were linked to carboxylic groups on the HRP enzyme in the presence of a soluble carbodiimide *(12)*. One milliliter of

fresh solution containing the enzyme (15 mg of HRP/mL; 100 m*M* EDC in 50 m*M* phosphate buffer, pH 6.0) was incubated with 50 mg of beads or 1.0-cm^2 PAN membrane (16 h at 22 ± 2°C). Immobilized enzyme preparations were washed and maintained as described earlier.

Quantification of Amine Groups

Primary amine contents of aminated PAN were measured by a modification *(13)* of the TNBS method *(14)*. The membrane samples were incubated in a 10 m*M* TNBS solution (in 100 m*M* borate buffer, pH 9.0) for 2 h at room temperature, becoming a strong orange. The matrices were then washed and submitted to an alkaline hydrolysis step (3 mL of 2 *M* KOH solution; 3 h at 45°C), regaining the original color. Primary amine contents were calculated by the amount of 2,6,4-trinitrophenol released to the alkaline solution (spectrophotometry at λ = 385 nm against a borate buffer blank; extinction coefficient of ε_{385nm} = 14,100 $M^{-1}\cdot cm^{-1}$).

Measurement of HRP Enzyme Activity

An enzyme reactor with an external loop was used in a batchwise mode to measure the immobilized HRP activity. This measuring system included a double-beam spectrophotometer (model U-2000; Hitachi, Japan) with an 18-µL glass cell of 1-cm optical length (Hellma, Germany), an external loop (300-µL total volume), a peristaltic pump (model 205S; Watson Marlow, UK), and a 10-mL glass vial (equilibrating vessel) magnetically stirred. Immobilized HRP-PAN was placed directly inside the vial. An immobilized HRP microbead packing column ($L \times \phi_{int} = 35 \times 1.5$ mm; of glass with cotton wool end stoppers) was interposed in the loop, downstream of the spectrophotometer.

The catalyzed condensation reaction between 4-AAP and PSA with H_2O_2 at room temperature was used to measure the HRP activity. The first two substrates were oxidized; H_2O_2 was reduced; and the formation of 4-benzoquinoneimine-2,3-dimethyl-1-phenylpyrazolin-5-one, the condensation product, was followed through its absorbance at λ = 490 nm. Absorbance readings were converted to H_2O_2 volumetric consumption using the condensation product extinction coefficient (ε_{490nm} = 6450 $M^{-1}\cdot cm^{-1}$). One unit of HRP activity (U) was defined as the enzyme needed to reduce 1 µmol of H_2O_2/min, on standard assay conditions.

A 5-mL aliquot of reaction medium without H_2O_2 (25 m*M* PSA and 0.4 m*M* 4-AAP in phosphate buffer) was circulated through the loop for absorbance baseline establishment. Next, 50 µL of soluble enzyme solution or insolubilized enzyme was inserted in the system (quantities compatible with linear variation in absorbance for 100–300 s). The flow rate (1.0 mL/min) was reestablished and 125 µL of 250 mg H_2O_2/L solution, previously standardized by acidic permanganate titration using oxalate standard solutions, was added to the vessel with the simultaneous start of 490-nm absorbance registration.

Enzyme kinetic studies were performed with all the HRP-derivatized supports. Nonactivated supports were also evaluated as controls.

Evaluation of Operational Stability of HRP

To evaluate the operational stability of HRP, the reactor system was used without the loop. A fresh reaction mixture (same as before but increasing the final H_2O_2 concentration to 8 mg/L) was continuously pumped through a packed column (or stirred 10-mL vessel) with the immobilized HRP. Similar flow rates (<1 mL/min, owing to time-dependent increased resistance to flow) were imposed and controlled throughout parallel runs. Absorbance measurements were performed semicontinuously, alternating the tubing of the reaction medium with the effluent from one of the reactors, connected to the spectrophotometric cell. Catalysis (35 h) was performed in eight cycles, feeding washing buffer to the supports during resting periods (at room temperature). The experiment ran for a total of 278 h.

Determination of Total Immobilized Protein

HRP-containing supports were washed with distilled water, withdrawn from the bioreactor, dried (48 h at 80°C), and weighted. Total immobilized protein was determined by a modified method of Lowry et al. *(15)* (100°C alkaline hydrolysis step, for 20 min). Calibration curves were prepared with BSA plus underivatized support (sample amount).

Results and Discussion

Derivatization of PAN

Measurements of primary amine contents on the nonderivatized UltraFilic membrane by the TNBS method yielded a background value of 3.3 ± 0.95 μmol of NH_2/mg of support dry wt. After activation with $LiAlH_4$ a value of 8.5 ± 3.3 μmol of NH_2/mg of support dry wt, i.e., 3×10^{19}–8×10^{19} NH_2/cm^2 of external surface, was reached using the same detection procedure. Higher concentrations of $LiAlH_4$ and/or higher reaction times were also used; however, the concentration of amine groups did not increase and membrane integrity was compromised. The content of amino groups of the PAN membrane is much higher than the densities of aminated silica and glass nonporous thin films ($\approx 4 \times 10^{13}$ NH_2/cm^2) *(8)*. Although the amino content of PAN was referred to the external surface, it is a high value and adequate for enzyme immobilization.

Activity of Immobilized HRP

The kinetics of condensation product formation by immobilized HRP was evaluated, in triplicate runs, for all the tested supports. From these data, the average initial rates of condensation product formation (initial activities) of immobilized peroxidase were calculated. An example of the time course of reaction is shown in Fig. 1 for HRP-glass by an EDC-medi-

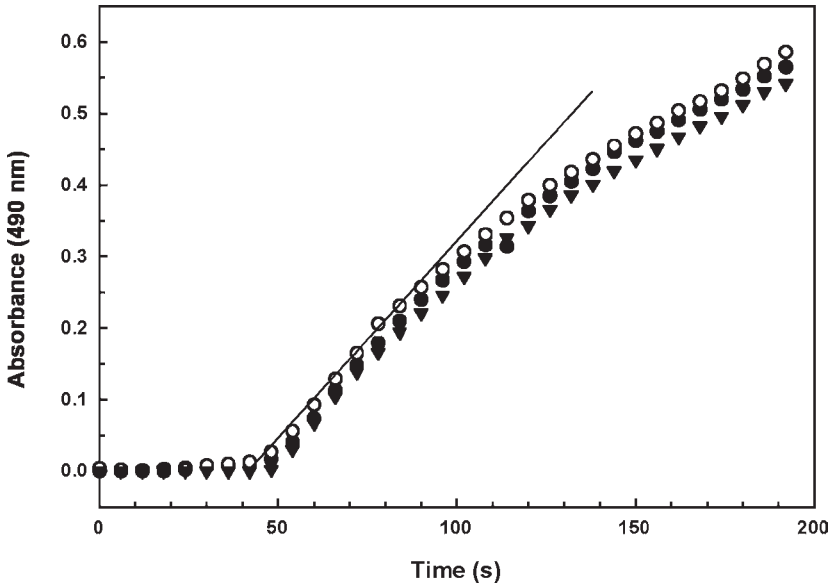

Fig. 1. Time course of reaction with immobilized HRP-glass-EDC. Data refer to triple experiments. (1st [●], 2nd [○], and 3rd [▼] runs.)

ated coupling chemistry. The HRP activities obtained for all matrices are given in Table 1.

Regarding the immobilized activities and specific activities obtained after the HRP insolubilization procedures, the best performance was observed with inorganic microbeaded supports. On these supports the HRP activity per support dry weight was one order of magnitude higher than that of HRP-PAN. The highest immobilized HRP activity (15.7–27.9 U/g of support dry wt) was obtained with the porous glass support.

Carbodiimide coupling of the enzyme on the inorganic supports led to much less coupled protein when compared with glutaraldehyde coupling, although the immobilization efficiency was increased; the enzyme molecules were much more active than in the case of glutaraldehyde coupling. The specific activity of the HRP-glass-EDC preparation was 55.8 U/mg of protein, whereas it was 0.906 U/mg for glutaraldehyde coupling. The loss of activity is often associated with a rigid conformation of the enzyme molecule after covalent linking through the glutaraldehyde, leading to stereochemical hindrance on formation of the enzyme-substrate intermediate complex *(11)*. This same phenomenon would be expected with the PAN support, but this was not verified. The nonderivatized PAN membrane control presented very high activities, probably owing to an inefficient membrane-washing step.

Calculated HRP immobilization yields, based on enzyme activity balances, were on the order of 30–40% (38% particularly for HRP-PAN-EDC), usual values for enzyme covalent coupling.

Table 1
HRP Activity and Respective Protein Content of Immobilization Supports

Immobilization support	Protein (mg/g support)	HRP activity U (μmol H_2O_2/min)[a]	HRP activity U/g support	HRP activity U/mg protein
PAN–control	0.050	0.021	1.38	27.6
PAN–glutaraldehyde	0.025	0.043	0.768	30.7
PAN–EDC	0.500	0.057	1.15	2.30
Silica–control	0.000	0.024	0.848	—
Silica–glutaraldehyde	13.1	0.034	1.35	0.103
Silica–EDC	0.050	0.058	2.44	48.8
Glass–control	0.000	0.063	3.75	—
Glass–glutaraldehyde	17.3	0.202	15.7	0.906
Glass–EDC	0.500	0.193	27.9	55.8

[a]Supports with different dry weights, varying from 7 (glass-EDC) to 55 mg (PAN-glutaraldehyde).

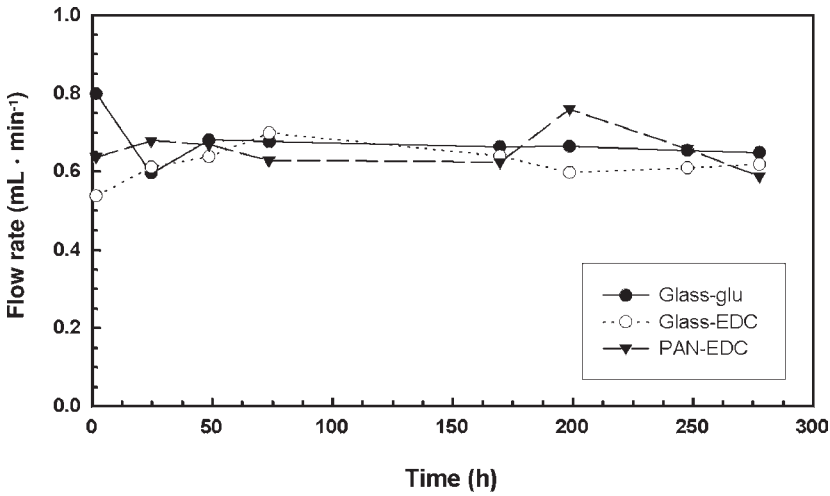

Fig. 2. Time variation of flow rate effluent from immobilized HRP reactors during operational stability studies.

Operational Stability of Immobilized HRP

The controlled flow rates of effluent reaction media of the three reactors and the respective volumetric depletion of H_2O_2, throughout the operational stability experimental runs, are shown in Figs. 2 and 3, respectively.

Glass microbeads, although providing a high initial specific activity of HRP with EDC activation (Table 1), are not adequate for long-term operation. The volumetric amount of oxidant reagent (H_2O_2) consumed in the stream effluent from the immobilized HRP-glass-EDC reactor was only

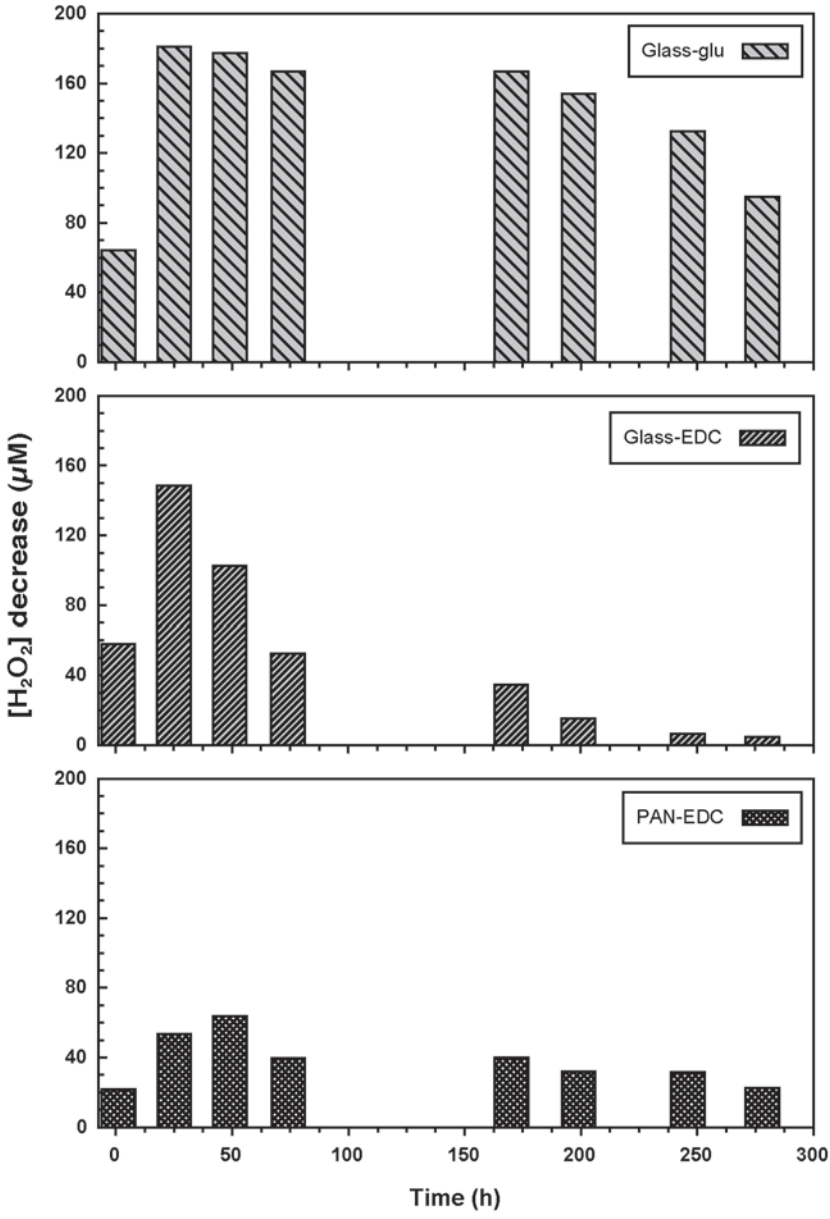

Fig. 3. Decrease in oxygen peroxide concentration in stream solution effluent from reactors with immobilized HRP during operational stability studies.

4.6 μ*M* after 278 h, much lower than 22.6 μ*M* obtained with the HRP-PAN-EDC system.

At the beginning of the operational stability studies, the amounts of active enzyme were different among the studied systems (owing to unequal dry mass of supports and coupling chemistries). This led to the use of different time scales of HRP turnoff, and data normalization was necessary

Table 2
Specific Productivity of Immobilized HRP
for Different Supports During Operational Stability Studies[a]

	Immobilization support		
Catalytic cycle	Glass–Glu	Glass–EDC	PAN–EDC
1	17.6[b]	12.6[b]	100
2	31.9	28.2	163
3	35.7	20.3	191
4	33.4	11.4	112
5	32.7	6.85	112
6	30.3	2.84	109
7	25.4	1.26	93.1
8	18.2	0.878	59.5

[a]Units of specific productivity are µmol of H_2O_2 consumed/(h·$U_{t = to}$).
[b]Enzyme reactors were unstable in this cycle (first 2 h) owing to compression of packed beads and its influence on flow rates.

for effective comparison. Therefore, the data in Figs. 2 and 3 were combined and converted into specific productivity of HRP-immobilized supports, based on the molar rate of reduced H_2O_2 per unit of initial activity of the respective immobilized peroxidase (Table 2).

After normalization of experimental data, it is clearly seen that UltraFilic membrane was the best support for long-term catalysis, retaining a higher specific productivity after 35 h of operation in a total period of 278 h at room temperature (Table 2). The final productivity (59.5 µmol of H_2O_2 reduced/[h·$U_{t = to}$]) with HRP-PAN was 3.3-fold higher than that verified with the HRP-glass-glutaraldehyde system. This is an interesting achievement because the glutaraldehyde-mediated enzyme coupling is usually recognized as the one leading to increased enzyme stability, owing to stabilization of enzyme conformation. Using glutaraldehyde-mediated HRP immobilization on alkylamine glass beads a high enzyme stabilization was reported *(9)*—81% residual activity of immobilized HRP after 6 mo on regular use of the beads. However, the overall duration of enzyme operation (or how often it was performed) was not indicated.

The very hydrophilic nature of the UltraFilic membrane may be responsible for the increased operational stability (Table 2) since the binding of water molecules in the microenvironment of the immobilized enzyme may ensure ideal conditions for maintenance of HRP native conformation. In addition, the hydrophilic character of supports is usually one of the most important criteria for selection of immobilization matrix *(11)*.

Another possible explanation for the high operational stability might be related to the involvement of carboxylic groups in the HRP binding to the support. Usually, HRP is linked to the support through the enzyme amine groups, leading to high immobilization yields. Carbodiimide-activated carboxylic residues on the enzyme can lead to the formation of

enzyme dimmers, or even multiple aggregates, by reaction with NH_2 in other protein molecules, compromising the yield of enzyme immobilization. Vianello et al. *(8)* confirmed the higher yields of HRP immobilized by their amine groups on glass and silica thin films as compared to immobilization through the carboxylic groups. However, operational stability data were not presented. Instead, a 40-d half-life time for HRP on carboxylic-activated glass and silica, kept at pH 7.0 and 6°C, was reported.

Conclusion

The successful immobilization of HRP in a hydrophilic, aminated PAN microfiltration membrane was demonstrated. A membrane with a specific HRP activity of 1.15 U/g of support dry wt was obtained by a carbodiimide–mediated coupling method. The specific activity was 2- and 24-fold lower than those verified for silica and glass aminopropyl supports, respectively, under identical carbodiimide-mediated immobilization processes. However, a higher operational stability was observed with the PAN-immobilized HRP system compared to other enzymatic supports. After an overall operation period of 35 h, the enzymatic membrane had a specific productivity of HRP of 59.5 μmol of H_2O_2 reduced/$(h \cdot U_{t=to})$. This value was 68- and 3.3-fold higher than the productivities of HRP-glass-EDC and HRP-glass-glutaraldehyde matrices, respectively.

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