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Immobilization of laccase on polymer grafted polytetrafluoroethylene membranes for biosensor construction

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ABSTRACT

In this study, *Trametes versicolor* laccase was immobilized on polytetrafluoroethylene (PTFE) membranes using two different techniques, entrapment to gelatin and covalent immobilization to the surface. For surface immobilization, functional groups were formed on PTFE surface by radiofrequency (RF) plasma treatment followed by polymer grafting. Two different polymers, polyacrylamide (pAAm) and polyacrylic acid (pAAc) were tried. For polyacrylamide grafted PTFE, a two-step polymerization process was used. The membranes were first treated with hydrogen plasma and pAAm grafted PTFE (pAAm-g-PTFE) was then formed by argon plasma treatment. To produce pAAc grafted PTFE (pAAc-g-PTFE), the surface was first treated with argon plasma and AAc was then attached to the surface by heat treatment (70 °C, 6 h). For both cases, an optimized carbodiimide coupling reaction was used for laccase immobilization. Enzyme activity was measured by an oxygen electrode using guaiacol as substrate. All three biosensing membranes were characterized and compared in terms of optimum working conditions, storage stability and reusability. Our study concluded that although a higher activity was obtained by gelatin entrapped laccase, its mechanical instability and poor storage life makes the gelatin biosensor unattractive for multiple usages and for field measurements. pAAc-g-PTFE biosensor was found to be more stable and highly reusable (ca. 50 times) when compared with the other two biosensors. In addition, its sensitivity was suitable for field applications. Therefore, the pAAc-g-PTFE biosensor could be proposed as an alternative on-site detection tool for phenolic compound monitoring.

1. Introduction

Highly toxic phenolic compounds are originated from both industrial and agricultural sources, namely textile, pulp and paper industry, coal conversion, resins, metal coating, petrochemicals, olive processing plants, partial degradation of phenoxy based herbicides and wood preservatives. Their presence in drinking and irrigation water poses a serious health risk [1] and some of these compounds have esterogenic and antiesterogenic activities that disrupt endocrine system [2]. Various biosensors based on tyrosinase [3–5], laccase [6–12] and peroxidase [13–15] enzymes and their mixtures [16,17] have been designed for the detection of these highly toxic compounds. Fungal laccases are commonly used in the construction of various biosensors [6,8,11,12,18]. A biosensor designed for toxic phenolic compound in environmental samples should be easy to use, portable and reusable with a reasonable storage stability. In this study, laccase enzyme was directly immobilized on PTFE membranes used in oxygen electrodes. PTFE is the material of choice in the oxygen electrodes because of its inertness, relative strength and temperature resistance. Another important advantage is that PTFE can be manufactured in the form of thin sheets (less than a micron) and is highly oxygen permeable. Immobilization of proteins on PTFE surface, on the other hand, requires the creation of reactive groups on the surface. This could be done by chemical [19] or physical methods, such as plasma treatment [20–23] or radiation-grafting [24].

In this study, radiofrequency (RF) plasma treatment was chosen for PTFE surface modification. RF plasma treatment is one of the easiest and common ways to treat polymeric surfaces due to its ability to produce a large volume of stable plasma. To be used as enzyme immobilization sites, amide and carboxyl groups were created on the surface by grafting two different monomers, namely acrylamide and acrylic acid. As a third approach, enzyme entrapment to gelatin matrix on the top of the PTFE membrane was used. The performance of all three biosensing membranes was compared. The aim was to construct an easy-to-use biosensing membrane which could be used with any oxygen electrode. Since the enzyme was not immobilized on an electrode but on the PTFE surface, this

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ready-to-use membrane could be sold or transferred to any area easily and then mounted to any oxygen electrode by an o-ring and used after calibration.

2. Material and methods

2.1. Materials

Laccase enzyme from *Trametes versicolor* (Sigma, 53739) was dissolved in phosphate buffer (pH 7.0, 0.1 M, 1 ml) and aliquoted into eppendorf tubes and stored at −20 °C until use. Guaiacol (Sigma, G-5202) solutions were prepared freshly every week. Apart from N-(3-dimethylaminopropyl)-n-ethyl-carbodiimide (EDC) (Fluka, 39391), and sodium dithionite (Merck, K38361007826), MES hydrate (M8250), N-hydroxy-succinimide (NHS, 130672) and glutaraldehyde (G7776) were purchased from Sigma–Aldrich Co. PTFE membrane supplied with oxygen electrode unit (Hansatech Instruments, Germany) was used as the immobilization support.

2.2. Methods

2.2.1. Determination of laccase activity

An oxygen electrode unit was used to measure the free and immobilized enzyme activity as described in Kok et al. [25,26]. This system enables the measurements in low sample volumes (1–1.5 ml) and provides an accurate temperature control via its water jacket. The calibration was done by adjusting the electrode reading to 0% in the presence of sodium dithionite and to 100% in vigorously oxygenated (30 min aeration) medium. The decrease in the oxygen amount originated from the enzyme activity was calculated by the software program provided by Hansatech Instruments (Germany).

2.2.2. Polymer grafting on PTFE surface

PTFE does not have any functional groups so it is unsuitable for enzyme immobilization unless it is modified. In order to immobilize laccase on PTFE surface, PTFE membranes were cut into 1.5 cm × 2.0 cm pieces, washed with acetone for 5 min to clean the surface from the contaminants and treated with radiofrequency (RF) plasma. Two different polymers, polyacrylamide and polyacrylic acid, were grafted on the surface via two different approaches.

2.2.2.1. Grafting of polyacrylamide (pAAm) to PTFE. PTFE samples were grafted with polyacrylamide by using a two-step protocol. The optimum conditions for maximum polymer grafting were determined in another study [27]. Shortly, the surface was first treated with hydrogen plasma (125 W, 13 Pa, 2 min) to increase surface wettability and treated samples were exposed to air for 2 h to generate the necessary reactive groups for polymer grafting. In the second step, 100 μl of acrylamide solution (25–50% (w/v) in 50% (v/v) acetone/ethanol) was spread over the samples and allowed to dry at room temperature for 15 min. Dried samples were treated with argon plasma (50 W, 13 Pa, 1 min) to initiate grafting of polyacrylamide onto PTFE. Treated samples were washed in dH_2O in orbital shaker overnight (200 rpm) to remove unbound polyacrylamide from the surface and dried in vacuum for 2 h at room temperature. Dried samples were analyzed with FTIR to confirm pAAm layer on the PTFE and new membranes were named as pAAm-g-PTFE.

2.2.2.2. Grafting of polyacrylic acid (pAAc) to PTFE. In order to graft polyacrylic acid, PTFE samples were first treated with argon plasma (50 W, 13 Pa, 1 min) and then exposed to air. The membranes were immersed in acrylic acid solution (30% in dH_2O and/or ethanol (v/v)) and heat activation was used to initiate the acrylic acid grafting. In this process membranes were kept in constant temperature (50–80 °C) for 6 h and then washed with dH_2O overnight in orbital shaker (200 rpm) to remove weakly bound polyacrylic acid from the surface. After the samples were dried in vacuum for 2 h at room temperature, the samples were analyzed with FTIR to confirm the pAAc layer on the surface and new membranes were named as pAAc-g-PTFE. The effect of temperature and solvent to grafting were investigated.

2.2.3. Construction of biosensing membrane

2.2.3.1. Gelatin entrapment. Gelatin (5, 7.5, 10 mg) was dissolved in 200 μl phosphate buffer (0.1 M, pH 6.0) at 38 °C. Before gelatin hardened, laccase enzyme (1 or 5 U) was added and the solution was placed on the surface of the PTFE and incubated at 4 °C for 1 h. When gelatin was dried, the membrane was immersed in glutaraldehyde solution (2.5, 5, 7.5%) for 4 min to crosslink the gelatin matrix. The construct was washed twice with dH_2O for 5 min to remove excess glutaraldehyde and unbound enzymes and stored at 4 °C until use.

2.2.3.2. Immobilization of laccase on pAAm-g-PTFE membranes. To immobilize laccase on pAAm-g-PTFE, carbodiimide–succinimide reaction was used. Amine groups on PTFE, which was generated by AAm grafting, was covalently bound to the carboxyl groups of the enzymes by using N-(3-dimethylaminopropyl)-n-ethyl-carbodiimide (EDC) and N-hydroxy-succinimide (NHS).

For covalent immobilization, 10–30 U of laccase enzyme was used. In order to determine the optimum amount of EDC to obtain maximum enzyme activity, different concentrations were tried. First, the amount of carboxyl groups on the enzyme was calculated and several EDC/carboxylic group (mol/mol) ratios were tested (from 0.05 to 0.6). For EDC/carboxylic group ratio of 0.1, for example, first the molarity of laccase enzyme was determined (for 10 U of laccase, carboxyl group amount is 7.350 μmol). EDC concentration to give the ratio of 0.1 was then calculated as 0.735 μmol and half of this amount (0.360 μmol) was used for NHS. The immobilization reaction was carried out in MES buffer (0.1 M, pH 5.5, 5 ml) at 4 °C with continuous shaking (o/n, 40 rpm). The samples were washed twice with dH_2O and stored at 4 °C until use.

2.2.3.3. Immobilization of laccase on pAAc-g-PTFE membranes. Acrylic acid is a carboxylic acid, thus pAAc-g-PTFE surface has free carboxyl groups. For this reason, first the membrane surface was modified in the absence of the enzyme and then activated surface was incubated with the enzyme. To do that, EDC was dissolved in PBS (5 mg ml^{-1}; 0.1 M, pH 7.4) and pAAc-g-PTFE was immersed in this solution at 30 °C for 1 h to activate the carboxyl groups present on the surface. After activation, the samples were washed twice with dH_2O to remove unbound EDC from the surface. To immobilize laccase, activated surfaces were immersed in PBS (0.1 M, pH 7.4) and 10–30 U of laccase was added to the solution. The immobilization reaction was carried out at 4 °C overnight with continuous shaking (40 rpm). The samples were washed twice with PBS (0.1 M, pH 7.4) and stored at 4 °C until use.

For both pAAm-g-PTFE and pAAc-g-PTFE, control membranes were prepared by omitting the EDC/NHS activation step to determine the extent of non-specific enzyme binding to the plasma treated surfaces. In short, after the polymers were grafted to the surface and ungrafted fraction was washed off, the membrane was directly incubated with laccase under the same conditions, washed and activity measurements were performed.

2.2.4. Characterization of biosensing membranes

2.2.4.1. Determination of optimum working temperature and pH. The optimum working temperatures of free and immobilized enzymes were determined by using oxygen electrode unit. The immobilized

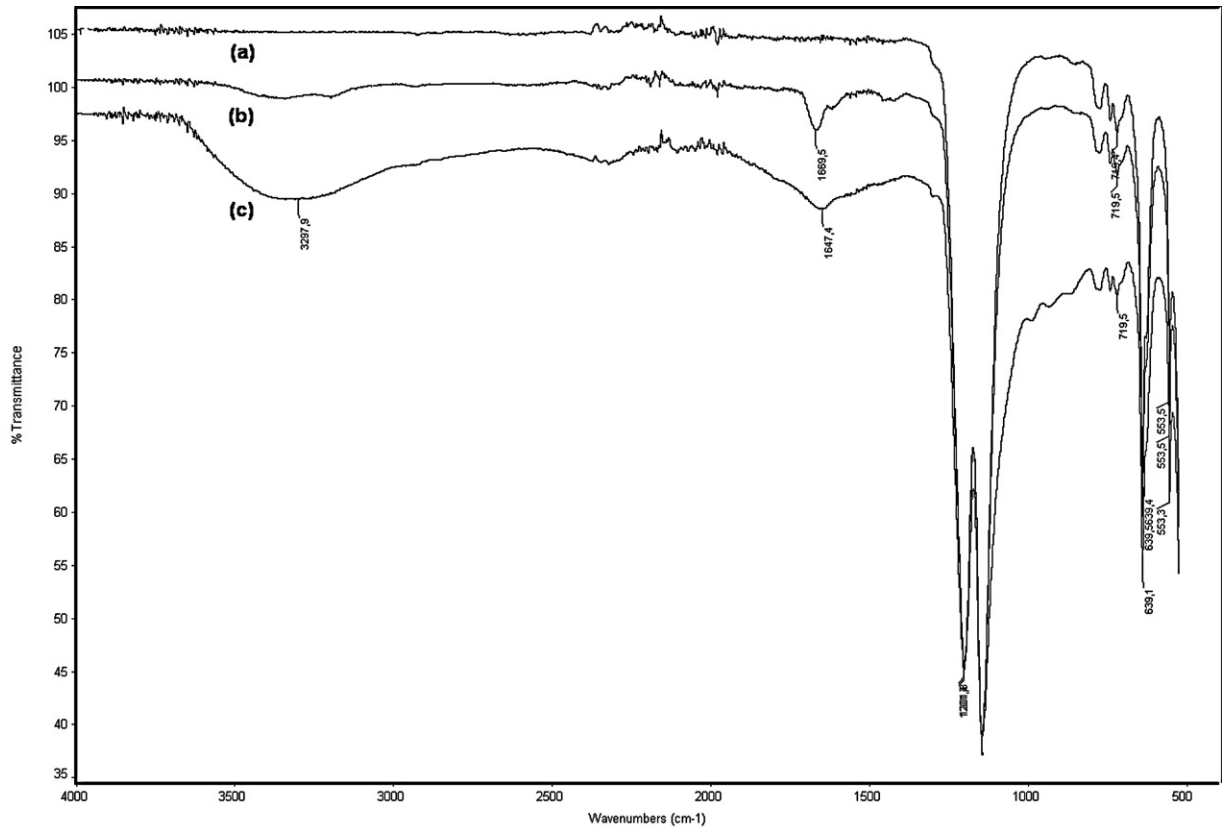

Fig. 1. ATR–FTIR analysis of (a) untreated, (b) acrylamide grafted and (c) acrylic acid grafted PTFE samples.

systems' activity was measured at temperatures between 25 and 50 °C. For the detection of optimum working pH, the activities of the constructs were measured between pH values of 3.5 and 7.0 by using 400 μM guaiacol as substrate.

2.2.4.2. Determination of reusability. In order to evaluate the reusability of the immobilized systems, the activities of the constructs were measured at optimum working conditions repeatedly by using guaiacol (400 μM) as substrate until the activity decreased to 60% of the initial activity.

2.2.4.3. Stability of immobilized systems. One of the main objectives of the immobilization is to extend the stability of the enzymes. Therefore, storage stability of the immobilized system is very important for a successful biosensor. To investigate the storage stability, thus the shelf life of the immobilized system, prepared constructs were stored at 4 °C in PBS. For each time point, two of these constructs were taken and their activity was measured at their optimum working conditions with 400 μM guaiacol.

3. Results and discussion

3.1. Optimization of the biosensor construction

Guaiacol (400 μM) was then chosen as the model analyte for both optimization and characterization studies since this concentration was in the linear working range for all three systems and it had a reasonable reaction rate.

3.1.1. Gelatin entrapment

3.1.1.1. Effect of gelatin amount. Different amounts of gelatin (5, 7.5 and 10 mg) were tested for laccase immobilization. Very low activities and insensitivity to the changes in substrate concentration were observed at 10 mg of gelatin, indicating a dense membrane causing diffusion limitation. 5 mg of gelatin entrapped system, on the other hand, lacked the mechanical stability needed for the application and ruptured easily when inserted into the oxygen electrode reaction chamber. It was possible to form responsive membranes with enough mechanical stability at 7.5 mg of gelatin so this concentration was used in further studies.

3.1.1.2. Effect of glutaraldehyde concentration. Three different concentrations of glutaraldehyde (i.e., 2.5%, 5%, 7.5%) were used to crosslink the gelatin. The results showed that the activity of the entrapped system decreased from 4.8 ± 0.8 to 4.2 ± 0.3 nmol ml^{-1} min^{-1} when glutaraldehyde concentration was increased from 2.5% to 5.0%. The decrease in activity was more pronounced (1.6 ± 0.1 nmol ml^{-1} min^{-1}) when glutaraldehyde concentration was further increased to 7.5%. This phenomenon could be the result of the increased crosslinking of the enzyme as well as the increased diffusion limitation for the substrate as a result of excessive crosslinking of gelatin. In this study, 2.5% of glutaraldehyde, which was found to give maximum activity with reasonable mechanical stability, was used in the further characterization experiments.

3.1.2. pAAm-g-PTFE immobilized system

Plasma induced grafting of pAAm on PTFE was confirmed using FTIR spectrophotometry. pAAm-g-PTFE samples showed a new peak at 1670 cm^{-1} corresponding to amide groups generated by pAAm grafting in addition to the peaks at 1200 cm^{-1} corresponding to C–F bonds in PTFE (Fig. 1b). Although 25% (w/v) acrylamide was successfully grafted on PTFE (confirmed with FTIR), the grafting yield was found to be too low for laccase immobilization. In other words, the laccase was immobilized on the grafted PTFE but the activity level was too low. More pronounced enzyme activities were

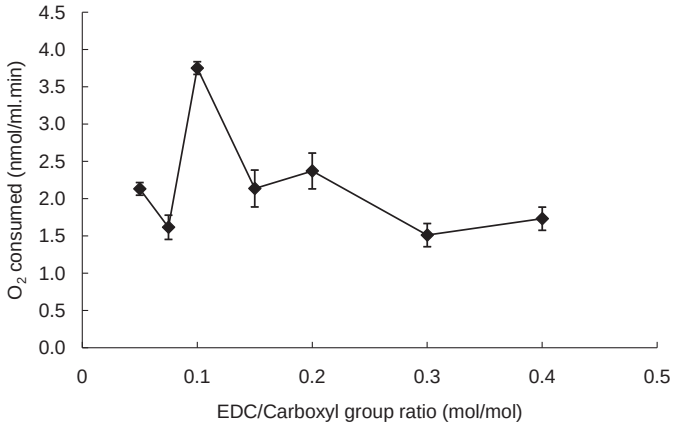

Fig. 2. Effect of EDC/carboxyl group ratio on laccase immobilization on pAAm-g-PTFE system.

obtained by using 50% (w/v) acrylamide. After successfully grafting the polymer, laccase immobilization was optimized by testing different NHS/EDC amounts and enzyme concentrations.

3.1.2.1. Effect of EDC/NHS amount. To covalently bind enzyme's carboxylic groups to the amine groups on the surface, EDC/NHS coupling reaction was used. To eliminate the risk of enzyme denaturation due to the excessive usage of EDC/NHS, this step was optimized. Several EDC/carboxylic group (mol/mol) ratios were tested (from 0.05 to 0.6) and the activity of the laccase immobilized surfaces measured in pH 6.0 PB at 35 °C by using 400 μM guaiacol (Fig. 2). It can be seen that NHS/carboxyl group ratio of 0.1 gave the maximum activity for enzyme immobilized system so it was chosen for further studies. When EDC/carboxylic group ratio was above 0.4, no activity was observed. Probably, the enzyme was negatively affected in high reactive chemical concentrations.

Some of the enzymes could be non-specifically adsorbed to the surface when they came into contact with the polymer grafted layer. In order to check this possibility, a control experiment was performed by omitting the EDC/NHS activation step as explained at the end of Section 2.2.3. No enzyme activity could be observed in this case. This shows that only when EDC–NHS activation step was introduced, a permanent bond that would not be affected from the washing step was established, and enzymes attached to the surface by weak interactions were washed off.

3.1.2.2. Effect of initial enzyme amount. To investigate the effect of initial enzyme amount on pAAm-g-PTFE immobilized system, 10, 20 and 30 U of laccase enzyme was used. The results showed a gradual increase in the immobilized enzyme activity (6.4 ± 0.4, 12.2 ± 1.8 and 20.2 ± 2.2 nmol ml^{-1} min^{-1}) with increasing initial enzyme amounts (10, 20 and 30 U). It could be seen that there is a linear increase in activity of the immobilized enzyme within the studied initial enzyme concentrations.

3.1.3. pAAc-g-PTFE immobilized system

In this immobilization method, the first step involving argon plasma treatment was needed to increase the wettability of the PTFE surface so that the acrylic acid solution could be evenly spread to the surface. Once this achieved, the second step involving grafting via heat activation was done. Even small amounts of oxygen in the medium resulted in failure in grafting, so the acrylic acid monomer solution was vigorously degassed and held under nitrogen atmosphere. Temperatures between 50 and 80 °C was investigated and 60 and 70 °C was found to be suitable for grafting. 50 °C was probably too low to initiate the polymerization. The failure in high temperature (at 80 °C) could be resulted from the competition of two different reactions during the process: polymerization of monomers in solution and grafting to the surface are competitive reactions and high temperatures may result in polymerization rather than the surface grafting [28], thus failure in grafting.

Another parameter affecting the grafting yield was the choice of solvent: although ethanol is an effective solvent for acrylic acid and successfully used in previous studies [29], our results showed that utilization of water led to more efficient results in terms of grafting, thus in enzyme immobilization. For further studies, argon treated PTFE was immersed in degassed 30% (v/v) AAc monomer solution and incubated at 70 °C for 6 h. FTIR results of the pAAc-g-PTFE membrane showed two new peaks, one at ca. 1650 cm^{-1} corresponding to C=O bonds and another broad peak between 2900 cm^{-1} and 3600 cm^{-1} (with a maximum at ca. 3300 cm^{-1}) corresponding to O–H bonds in AAc structure (Fig. 1c).

3.1.3.1. Effect of EDC/NHS amount. In this system, EDC/NHS concentration was not optimized as in the case of pAAm-g-PTFE membrane. In the pAAm-g-PTFE system, the carboxyl groups of the enzyme were activated so that excessive usage of the chemicals which would cause denaturation of the enzyme could be avoided. For this reason, an optimum value was found and presented in Section 3.1.2. pAAc-g-PTFE membranes, however, was activated in the absence of the enzyme so there was no chance of enzyme denaturation. Thus, the activation was performed with higher EDC concentrations at 30 °C for a rapid reaction. Omitting EDC/NHS step led to the membranes without any activity for the pAAc-g-PTFE membranes as in the case of pAAm-g-PTFE.

3.1.3.2. Effect of initial enzyme amount. Different starting enzyme concentrations (10, 20, 30 U) were tested for their effect on the final pAAc-g-PTFE membrane activity. The results showed that there was an increase in the immobilized enzyme activity (7.4 ± 0.5, 11.1 ± 2.1 and 13.5 ± 1.9 nmol ml^{-1} min^{-1}) with increasing initial enzyme amounts (10, 20 and 30 U). In this case, the increase is not linear but seemed to reach to a saturation value. The immobilized protein was, therefore, determined to check if this trend was originated from the immobilized enzyme amount or from another reason. It was seen that tripling the initial enzyme concentration did not increased the immobilized enzyme activity in the same degree. Although there was an increase, the yield (activity of immobilized/initial) dropped from 37.5% to 28.0% and finally to 23.7% as the enzyme amount increased from 10 to 30 U. In order to keep the yield higher and prevent unnecessary enzyme lost, 10 U of enzyme was chosen for further studies.

3.2. Characterization and comparison of immobilized systems

3.2.1. Effect of pH on free and immobilized laccase

The effect of pH on free and immobilized laccase was measured for guaiacol (400 μM) and the results were normalized by taking the highest activity of each immobilized system as 100% for a better comparison between different methods (Fig. 3). The optimum pH of free laccase was found to be pH 5.0, but a shift to alkaline or acidic values was observed for immobilized systems. For gelatin entrapped system, the optimum pH shifted to an acidic value (pH 4.0) while for covalently immobilized systems, a more alkaline value was observed (pH 6.0). This phenomenon is generally the result of the proton exchange properties of the immobilization matrix [30]. Immobilization matrix creates a microenvironment around the enzymes and this microenvironment has different characteristics than the bulk solution. Due to their different isoelectronic point values, the polymers may be charged positively or negatively at different pH values. Gelatin is a positively charged matrix and because of this, the pH of its microenvironment is higher

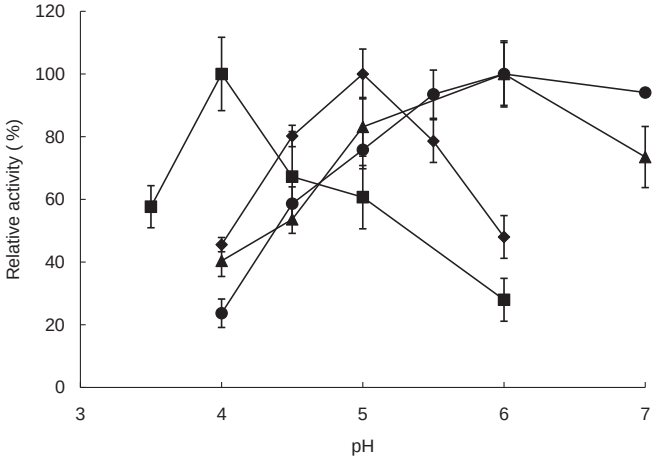

Fig. 3. Effect of pH on free and immobilized laccase activity. The lines correspond to laccase activity in (♦) free, (■) gelatin entrapped and covalently immobilized (on (▲) pAAm-g-PTFE and (●) pAAc-g-PTFE) forms using guaiacol (400 μM) as substrate. The results are the average of at least three measurements.

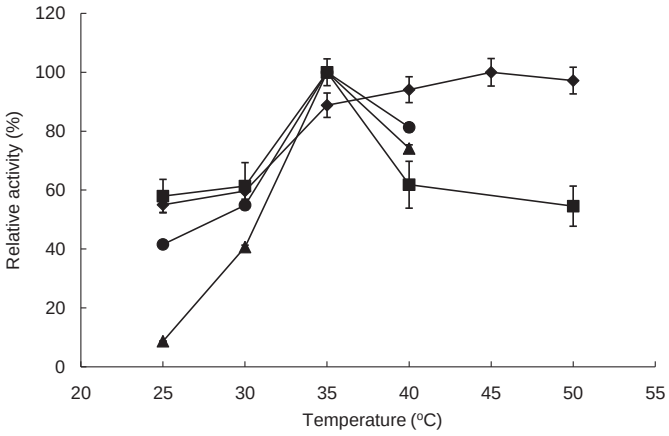

Fig. 4. Effect of temperature on free and immobilized laccase activities. The lines correspond to laccase activity in (♦) free, (■) gelatin entrapped and covalently immobilized (on (▲) pAAm-g-PTFE and (●) pAAc-g-PTFE) forms using guaiacol (400 μM) as substrate. The results are the average of at least three measurements.

than the bulk pH. For example, when pH was measured as 4.0 in the bulk solution, in the enzyme's microenvironment, it shifted to more basic values, may be at around 5.0.

The optimum pH of the pAAm-g-PTFE and pAAc-g-PTFE immobilized systems was found to be 6.0. Unlike the gelatin entrapped system, optimum pH shifted towards higher values. In this case, both polyacrylamide and polyacrylic acid are anionic polymers, thus the microenvironment pH value is lower than bulk pH. Although both systems showed similar responses to pH changes, it could be seen that the pAAc immobilized system was more stable in neutral pH values. Different buffering capacities of the polymers could be the cause for this effect.

It could be seen that gelatin entrapped system had a narrower working pH range, i.e., the immobilized enzyme system kept more than 60% of its activity only in pH values between 3.5 and 5.0 (Fig. 3). pAAm-g-PTFE and pAAc-g-PTFE immobilized systems, on the other hand, conserved more than 60% of their activity in between pH 4.5 and 7.0. This could be considered as an advantage since it allows the biosensing membrane to be used in the measurement of various real samples at different pH values without a pronounced activity loss.

3.2.2. Effect of temperature on free and immobilized laccase activity

To determine the optimum working temperature of free and immobilized laccase, measurements were done at different temperatures using 400 μM guaiacol. For each system, its optimum working pH value was used. The results showed that the activity level of free laccase was above 90% at temperatures between 35 and 50 °C and the maximum activity was reached at 45 °C (Fig. 4). The impact of temperature change was found to be more pronounced in immobilized systems. Maximum activity was observed at 35 °C for all immobilized systems. The activity of pAAm-g-PTFE immobilized system dropped drastically at lower temperatures with 60% activity loss at 30 °C and almost no activity was observed at 25 °C. Gelatin entrapped system, on the other hand, was sensitive to high temperatures and 40% activity loss was detected with a 5 °C increase in temperature. Gelatin starts to dissolve above 40 °C and this was the reason for the activity loss after 35 °C. Gelatin entrapped enzyme systems had been reported to show similar activity loss after 35 °C [9,31]. The most thermally stable system was found to be the pAAc-g-PTFE immobilized one.

3.2.3. Reusability of the immobilized systems

The main advantage of immobilization is the ability to reuse the same system with minimal or no loss of activity. To determine the reusability of the constructs, the same immobilized enzyme system was repeatedly used and activities were determined (Fig. 5). Since gelatin entrapped construct was not mechanically stable, the membrane was ruptured after 3–4 uses. This resulted in enzyme leakage and rapid loss of activity; thus, the results are not included in Fig. 5. Covalent immobilization of laccase on pAAm and pAAc grafted PTFE resulted in a distinct advantage in terms of reusability. pAAc immobilized system was found to be more stable than the pAAm immobilized construct. The pAAm immobilized construct lost 40% of its initial activity after 35 consecutive uses. The pAAc immobilized construct, on the other hand, reached the same percent of activity loss after 50 consecutive uses. Reusability of the laccase biosensors has not been reported in most of the studies. Roy et al. [11] have found a 40% of activity lost after 30 measurements for their biosensor based on crosslinked laccase crystals (CLEC). Although CLEC has an advantage of good stability concerning temperature, solvent and storage conditions, colored products during the reaction have been observed to accumulate on the matrix, causing its inactivation with time. A laccase–tyrosinase dual biosensor was found to maintain only about 13% of its initial activity after 60 measurements [17]. It could, therefore, be said that the biosensor developed in this study has a comparable,

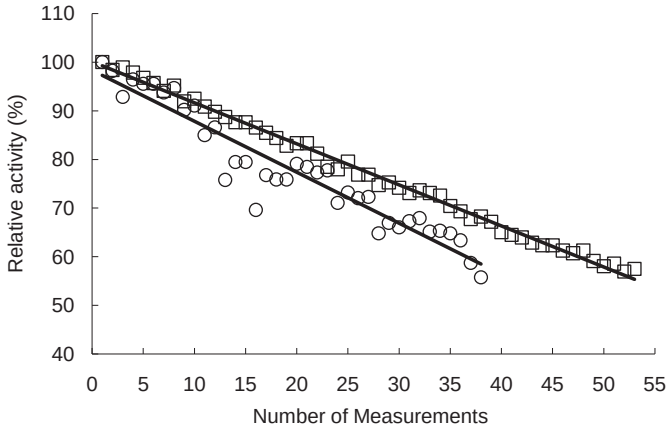

Fig. 5. Reusability of laccase immobilized on (□) acrylic acid and (○) acrylamide grafted PTFE membranes. Reusability of gelatin entrapped biosensor was limited to 3–4 times so it was not included in the graph.

Table 1
Comparison of the constructed biosensors in terms of analysis conditions and biosensor properties (400 μM guaiacol was used as substrate).

	Gelatin entrapment	pAAc-g-PTFE	pAAm-g-PTFE
Preparation time	2 h	1.5 days	1 day
Optimum pH	4	6	6
Optimum temperature (°C)	35	35	35
Activity (nmol ml^{-1} min^{-1})	12.0	7.4	6.4
Minimum detection limit[a] (for guaiacol, in μM)	82.1	91.3	125.1
Reusability[b]	3–4	ca. 50	ca. 35
Storage stability (days)[c]	13	20	6

[a] The minimum detection limit was the guaiacol concentration which gave 2 nmol ml^{-1} min^{-1} of activity.
[b] The biosensor was considered to be reusable until it lost 40% of its original activity.
[c] The time at which biosensors lost 60% of their original activities.

even better, reusability than its counterparts found in the literature.

Fig. 5 could also give information about the reproducibility of the signal. It could be seen that the signal fluctuations were in minimum in the biosensor prepared by pAAc-g-PTFE. The fluctuations were more pronounced in pAAm-g-PTFE biosensor.

3.2.4. Stability of the immobilized systems

The stabilities of immobilized systems in PB (pH 6.0, 0.1 M) at 4 °C were investigated (Fig. 6). pAAm-g-PTFE immobilized system found to be the most unstable construct; it lost 60% of its initial activity after 6 days. The pAAc immobilized system and gelatin entrapped system, on the other hand, lost the same amount of activity only after 20 and 13 days, respectively. Storage stability was not reported for many of the laccase and/or tyrosinase biosensor studies. Kim and Lee [32] tested the wet storage stability of their silicate and silicate/Nafion based tyrosinase biosensor and found that the activities dropped to 50% and 74% of its original value, respectively, in a 2-weeks period. In another study, the stability of the laccase/Nafion electrode was determined at room temperature [33]. It was seen that the introduction of gelatin increased storage stability of the laccase/Nafion electrode and 80% of the initial activity retained instead of 60% after 5 days of storage. These rapid decreases in activity were probably the result of the wet storage. A suitable drying technique, preferably approaches such as freeze-drying could be a better alternative for storage of the protein related systems to decrease the activity loss.

3.2.5. Overall comparison of three immobilization methods

Table 1 summarizes the properties of three different immobilization methods that were studied to construct the laccase biosensor for the detection of toxic phenolic contaminants. For each method, PTFE was used as the immobilization support. The first method involving gelatin entrapment on the membrane surface had an advantage in terms of ease of preparation and higher activity while its poor mechanical stability led to a limited reusability. Laccase immobilized on polyacrylic acid grafted membranes (pAAc-g-PTFE), on the other hand, gave the best reusability and storage stability results, especially when compared with the enzyme immobilized on pAAm-g-PTFE membrane. For both methods involving covalent immobilization of laccase, optimum working pH was shifted to more neutral values and the enzyme activity was preserved over a wider range of pH. This could be considered as another advantage over gelatin-entrapped system in which a narrower pH activity profile was observed. Narrow pH activity range could be a problem while working with real samples in the field. Minimum detection limits for guaiacol were found to be similar for gelatin entrapped system (82 μM) and pAAc-g-PTFE (91 μM) while pAAm-g-PTFE resulted in a slightly less sensitive system (125 μM).

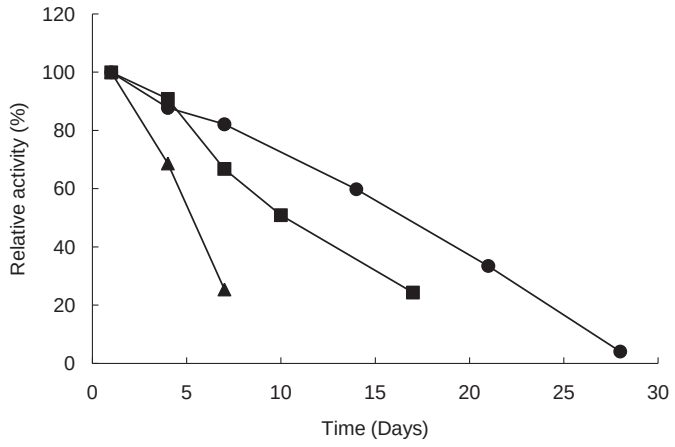

Fig. 6. Stability of constructed biosensors. The lines correspond to percent residual activity for (■) gelatin entrapped and covalently immobilized (on (▲) pAAm-g-PTFE and (●) pAAc-g-PTFE) laccase in PB (pH 6.0, 0.1 M) at 4 °C.

4. Conclusion

Construction of an easy-to-use enzyme biosensor for on-site detection of toxic phenolic compounds was aimed and for this, three different immobilization methods were optimized and compared. Covalent immobilization of laccase on pAAc-g-PTFE was found to be the best method for biosensor construction in terms of reusability and storage stability. Its minimum detection limit is better than pAAm-g-PTFE and comparable to gelatin entrapped one. Immobilization of laccase on PTFE membrane, in stead of the direct immobilization on electrode surface as usually done, allowed us to obtain a ready-to-use biosensing membrane with high reusability. This membrane could be transferred to any place easily, then mounted on any oxygen electrode by an o-ring and used after calibration.

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