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Layer-by-Layer assembled laccase enzyme on stimuli-responsive membranes for chloroorganics degradation

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Abstract

Functionalized membranes provide versatile platforms for incorporation of bio-catalysts and nanostructured materials for efficient and benign environmental remediation. The existing techniques for remediating chloro-organics in water consist of both physical and chemical means mostly using metal oxide-based catalysts, despite associated environmental concerns. To offer bioinspired remediation as an alternative, we herein demonstrate a layer-by-layer approach to immobilize laccase enzyme into pH-responsive functionalized membranes for degradation of chloro-organics from water. Efficacy of these bioinspired membranes towards dechlorination of 2,4,6-trichlorophenol (TCP) is demonstrated under pressure driven continuous flow mode (convective flow) for the first time to the best of our knowledge. Over 80% of the initial TCP was degraded at optimum flow rate under an applied air pressure of about 0.7 bar or lower. This corresponds to degrading a substantial amount of the initial substrate in only 36 seconds residence time, which in a batch reaction take hours. This, in fact, demonstrates an energy efficient flow through system with potential large scale applications. Comparison of the stability of the enzyme in solution phase vs. immobilized on membrane phase showed a loss of some 65% of enzyme activity in the solution phase after 22 days, whereas the membrane-bound enzyme lost only a negligible percentage of activity in comparable time span. Finally, the membrane was exposed to rigorous cycles of TCP degradation trials to study its reusability. The primary results reveal a loss of only 14% of the initial activity after four cycles of use in a period of 25 days, demonstrating its potential to reuse. Regeneration of the functionalized membrane was also validated by dislodging the immobilized enzyme followed by immobilization of fresh enzyme on to the membrane.

Keywords

Functionalized Membrane; Layer-by-Layer Assembly; Enzyme Immobilization; Laccase; Catalysis; Trichlorophenol

INTRODUCTION

Increasing demand for new approaches of water disinfection, decontamination and desalination mandate developments in membrane technology advancing the utility of

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Supporting Information. Michaelis-Menten Plots, UV-Vis Spectra, Chromatogram showing Degradation of TCP, Mass Spectrum from LC-MS; this material is available free of charge via the Internet at <http://pubs.acs.org>.

synthetic membranes for water treatment.^{1–2} Recently-developed functionalized membranes with porous support and functional polymer matrices provide the possibility of tuning the membrane pore structure, selectivity, and reactivity.^{3–5} Moreover incorporation of stimulus-responsive materials into the pores expands the applicability of the membranes into areas such as separation, biosensors, delivery systems etc.^{6–8} Introduction of functional polymers into the membrane pores enables deliberate immobilization of biological molecules such as enzymes within the polymer matrix, thus allowing catalytic remediation of toxic organics in water. Apart from providing enhanced stability, reusability and ease of handling, immobilization of enzymes onto membranes increases their practical utility by coupling them with immediate separation of the products in a flow-through system thereby minimizing inhibition of enzyme activity.^{9–10}

Chlorinated organic compounds such as chlorophenols are a class of persistent environmental pollutants which have been extensively used as wood preservatives and for pesticide formulation over the years.¹¹ These chlorinated organic compounds have a long history as threats to human health. Various chloro-organics, such as, 2,4,6-trichlorophenol (TCP), have immense health impact, and is widely detected in ground water and soil.^{12–13} Existing remediation techniques degrade the chloro-organics by both physical and chemical methods.^{14–15} In contrast, bioremediation is accomplished using enzymes which are environmentally benign, renewable and non-toxic.^{16–17} Furthermore enzymes are highly selective natural biocatalysts that accelerate reactions with extreme efficacy and selectivity. However, enzymes have had limited industrial application due to their lack of long term operational stability, intricate recovery and poor re-usability. Fortunately, these limitations can often be overcome by immobilizing them on suitable matrices such as functionalized membrane.^{18–19}

Laccase has long been used in the paper industry for its lignolytic activity, and also gained attention for its ability to degrade phenolic and other compounds in contaminated effluents.^{20–24} We therefore sought to combine promising activity of laccase with the versatile tunable behavior of polyacrylate polymers to fabricate bioinspired membranes for dechlorination of polychlorophenols. Our hypothesis is that under convective flow the reactants can rapidly access the active sites and the metabolized products leave the membrane and hence the enzyme active sites immediately after the reaction, eliminating unnecessary deterioration of the enzyme activity. To this end we report the immobilization of laccase on polyacrylic acid (PAA) functionalized polyvinylidene fluoride (PVDF) microfiltration membranes following a layer-by-layer (LBL) assembly technique.^{25–27} A general believe is that, unlike enzymes entrapped in matrix, covalent binding usually leads to higher stability due to increased enzyme-support attachment, however, reduces enzyme activity due to changes in the enzyme's native structure.^{28–32} LBL assembly offers such entrapment with an oppositely charged polyelectrolyte within the membrane domain without deteriorating the core structure. It helps to retain the enzyme activity without compromising on the stability aspect. Moreover past studies from our group have shown that it is possible to reload the enzyme when necessary.³³ The main aim of the current study is to understand the activity of such laccase-functionalized membranes towards degradation of polychloro-organics. Within that context, our specific goals are to (i) fabricate functionalized membrane by alternating attachment of cationic and anionic polyelectrolytes via the LBL technique and characterize

the functional domain, (ii) compare the stability and reactivity of the immobilized enzyme to those of the free enzyme, and (iii) evaluate the long term performance of the laccase-functionalized membranes towards degradation of 2,4,6-trichlorophenol in water under convective flow conditions. Activity of the fabricated bio-inspired membranes towards dechlorination of 2,4,6-trichlorophenol, as a model compound, is demonstrated. Degradation of up to 83% of the initial feed concentration has been achieved under pressure driven flow (convective) operation.

MATERIALS AND METHODS

Materials

All chemicals used during the laboratory-scale membrane fabrication and other studies were reagent grade and used without further purification. 2,4,6-trichlorophenol (TCP, 98%), Acrylic acid (AA, 99%), ammonium persulfate (APS, >98%) and the enzyme laccase from *Trametes versicolor* (powder, light brown, 0.5 U/mg) were purchased from Sigma Aldrich. Poly(allylamine-hydrochloride) (PAH) and N, N'-methylenebisacrylamide (MBA, >99%) were obtained from Acros, New Jersey, NJ, USA. Bradford reagent was obtained from VWR.

The PVDF microfiltration membranes used for all the experiments were supplied from Nanostone/Sepron Membranes Inc., Oceanside, CA (PAA-PV400HA and PV700, pore diameter 200–450 nm, thickness of ~125 μm).

Membrane functionalization

PVDF membranes were functionalized via *in situ* polymerization method as reported earlier.³⁴ Specifically, membrane functionalization was achieved via free radical polymerization of the AA at the PVDF pores (Scheme 1). A monomer solution of AA (20 wt % aqueous solution) with MBA as cross-linker (1.0 mol % of AA) and APS (1.0 mol % of AA) was embedded into the pores of the membrane by convective flow through it. The membranes were then baked at 70 °C for 1 h under N₂ atmosphere, then washed thoroughly with DIUF (Deionized ultra-filtered water) water to eliminate any unreacted constituents, dried and then weighed to confirm polymerization through the mass gain. The functionalization was further confirmed through conventional spectroscopic and microscopic (SEM) analyses as well as based on their water permeability as described below.

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Varian 7000E) was used to assess the presence of functional groups in the functionalized PVDF membranes. The samples were placed on a diamond crystal and the spectrum was obtained between 500 and 4000 cm^{-1} averaging 24 scans at a resolution of 4 cm^{-1} . The surface morphology of functionalized membranes was characterized via scanning electron microscopy (SEM, Hitachi S4300). Membrane porosity was then estimated as the ratio of pore area to total membrane area based on SEM images of the membrane top surface. The chemical composition of the functionalized membrane was studied with X-ray photoelectron spectroscopy (K-Alpha, Thermo-Scientific). Zeta potential analysis was performed with the Anton-Paar Surpass Electrokinetic Analyzer to characterize membrane surface charge.

Contact angle measurements for the functionalized membranes and bare PVDF membranes were made in a Kruss Drop Shape Analyzer instrument (DSA100).

The water permeability behavior of the PAA functionalized PVDF membranes was studied using a laboratory-scale stainless steel pressure cell (Sepa ST, GE Osmonics, effective membrane area is 13.2 cm²) in dead-end mode.⁴ Water flux was measured at four different pressures with at least three replicates at each pressure. For pH-responsiveness studies, DIUF water at different pHs was passed through the membrane until the permeate pH was identical to the feed pH.

Electrostatic immobilization of laccase in functionalized membranes

Laccase was immobilized into the pores of the PAA functionalized PVDF membrane by layer-by-layer assembly technique as demonstrated in scheme 1. PAA having a pK_a value of ~5 remains negatively charged after treatment with DIUF (pH 6). In order to embed a positively charged electrolyte into the membrane pores, 100 mL of a 43 μ M solution of PAH (in DIUF) was permeated through the membranes at pH 3.9. The electrostatic interaction between the negatively charged carboxylic acid groups of PAA and the positively charged ammonium groups of PAH holds the PAH immobilized into the pores. After formation of the PAA-PAH layers, laccase was immobilized electrostatically by permeating the membrane with 100 mL of a laccase solution (concentration varying from 50–150 ppm) in DIUF at pH 6. Laccase having a pI of ~3.5 is understood to attach electrostatically to the positively charged ammonium groups. As expected after immobilization of the PAH layers followed by the enzyme layers the flux of the membrane decreases significantly (by ~73%). The lower permeability with the laccase immobilization is desirable to achieve optimized residence time for effective degradation. The amount of laccase immobilized was determined by the difference in activity of the enzyme between the feed solution and the enzyme left in the permeate at the end of the immobilization. After the enzyme immobilization step, membranes were rigorously washed with DIUF water under convective flow mode in order to wash away any loosely bound enzymes. The washings were carried out with several 20 mL aliquots of DIUF water until the permeate is free of laccase enzyme. In general, the quantity of leached enzyme is negligibly low after the 3rd or 4th washing as quantified by Bradford assay (please see supporting information, Figure S2a). For cross verification purpose, aliquots from permeate were also tested for the presence of any enzyme by means of its reaction with ABTS. It should also be noted here that both these methodology for enzyme detection were accurate to use under these conditions; no color change of Bradford reagent or ABTS solution detected upon addition of either TCP or PAH only.

Bradford assay

The amount of the loosely bound enzyme leached out from the membrane with washing was quantified spectrophotometrically at 595 nm by means of its interaction with Bradford reagent. The protocol followed was as per the datasheet provided by VWR. The standard curve was obtained with BSA protein in the linear range of (0 μ g/mL – 10 μ g/mL). For each particular analysis 1 mL Bradford Reagent was added to each standard dilution (of protein/enzyme) with specific volume of 0.15 M NaCl solution and mixed it for 5 minutes before measuring the absorbance at 595 nm.

Assay of laccase activity

The activities of free and immobilized laccase was determined by monitoring the oxidation of 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) spectrophotometrically at 420 nm ($\epsilon_{\text{max}} = 36000 \text{ L mol}^{-1} \text{ cm}^{-1}$) with 0.2 mM ABTS as substrate in 0.25 M potassium phosphate buffer (pH 5.7) at 22 °C. The kinetic parameters, K_m and k_{cat} , were determined by measuring the initial rates of reaction between laccase and different concentrations of ABTS as the substrate (0.01–0.2 mM) in 0.25 M potassium phosphate buffer (pH 5.7) at 22 °C. The kinetic parameters were estimated from the Michaelis–Menten equation.

Performance of the laccase immobilized membranes towards degradation of TCP

TCP degradation studies were performed under pressure driven flow using a laboratory-scale stainless steel pressure cell housing the functionalized membrane. In a typical degradation experiment 100 mL of 250 μM solution of TCP in DIUF (pH 5.6) was allowed to pass through the membrane and the flow rate was varied under positive pressure from an air gas tank. The collected permeate was then analyzed for the concentrations of TCP left using high-performance liquid chromatography (HPLC) with a Shimadzu instrument employing two pumps, an auto sampler, and a photodiode array detector. Standard solutions of TCP were prepared to obtain a standard curve with which to quantify TCP degraded upon passage through the membrane. The elution was performed by pumping acetonitrile and water (55:45 v/v) at a flow rate of 0.6 mL/min. UV light absorption was measured over a wavelength window of 200–700 nm. The amount of TCP degraded by the laccase functionalized membrane was calculated using the following equation:

$$C_{\text{deg}} = C_{\text{feed}} - C_{\text{per}}$$

where, C_{deg} is the amount of TCP degraded by laccase; C_{feed} is the initial concentration of TCP in the feed solution; C_{per} is the amount of TCP in the permeate.

RESULTS AND DISCUSSIONS

Characterizations of the functionalized PVDF membrane

In order to assess the success of polymerization of PAA in the pores of PVDF membranes, the functionalized membranes were characterized by ATR FT-IR, SEM, Contact angle measurement, XPS, Surface Zeta potential measurement, and water permeability studies.

ATR FT-IR spectral analysis

ATR FT-IR spectra of PVDF and functionalized PVDF membranes attest to successful fabrication of the membranes. Figure 1 compares a PVDF membrane as supplied, a PAA-PAH functionalized PVDF membrane, and a laccase-functionalized PVDF membrane. The absorption peaks characteristic of fluorocarbon groups ($-\text{CF}_2-$) of the PVDF chains lie in the region of 1050–1280 cm^{-1} , as expected, for all the membranes.³⁵ Successful polymerization of PAA in the membrane matrix is revealed by the appearance of new peaks at 1717 cm^{-1} and 1544 cm^{-1} (Figure 1, black line) corresponding to the carbonyl stretch and antisymmetric stretching of carboxyl groups ($-\text{COOH}$), respectively, of the polyacrylic acid

polymer.³⁶ The FT-IR spectrum of free laccase is known to include two absorption bands, near 1645 cm^{-1} and 1540 cm^{-1} corresponding to the peptide backbone amide I and amide II modes, respectively.³⁷ Recorded spectra for laccase-functionalized PVDF membranes showed similar peaks (Figure 1, blue line). The peak at 1642 cm^{-1} was due to the stretching vibration of the C=O amide bond and the 1545 cm^{-1} peak was assigned to the combination of bending vibration of the N-H bond and stretching vibration of the C-N bond of the peptide backbone, signifying immobilization of laccase on the membrane support.²³ Moreover the presence of more peak features in the range $3500\text{--}3300\text{ cm}^{-1}$ suggests the presence of the amine groups belonging to the protein.

SEM imaging

The morphologies of the PVDF and PAA functionalized PVDF membranes were characterized by Scanning Electron Microscopy (SEM) as shown in Figure 2. The bare PVDF membrane (Figure 2a) shows a fairly porous structure with an average pore size of $192 \pm 50\text{ nm}$. The diameter and morphology of the membrane pores changed substantially after the PAA polymerization reaction, becoming uniformly circular and smaller with an average pore size of $112 \pm 5\text{ nm}$ (Figure 2b). These direct observations of pore diameter shrinkage demonstrate that PAA has polymerized into the pores. On successive modification of the membrane by immobilizing PAH and the enzyme the average pore diameter further reduced to $70 \pm 12\text{ nm}$ as can be seen in the figure 2c.

Contact angle measurement

To quantify any change in the hydrophilicity of the membranes, we compared the contact angle of water on functionalized membranes to that on bare PVDF membrane. The contact angle decreased from 80° to 56° indicating that membrane became more hydrophilic upon functionalization with the PAA polymer. This is consistent with the other spectroscopic characterizations of the membranes in indicating successful functionalization. As expected, the contact angles did not change significantly (Table 1) upon further modification of the membranes by putting in PAH and laccase in to the pores.

XPS analysis of functionalized membranes

The surface compositions of bare PVDF membranes, PAA-functionalized PVDF membranes and laccase immobilized PVDF-PAA membranes were explored by X-ray photoelectron spectroscopy (Figure 3). Survey spectra of these membranes show that the functionalized PVDF membrane (Figure 3b) contains significant N and considerably higher C content than the bare PVDF membrane (Figure 3a). The N content of the functionalized membrane is explained by the cross linker, *N,N'*-methylenebisacrylamide (MBA) used during polymerization. Deconvolution of the C1s peak near 290 eV for the functionalized membrane yielded three peaks corresponding to carbon in C-C (285 eV), C-O (287 eV), and the C=O (289 eV) functionalities (Figure 3c), in addition to a signature of C-F attributed to the PVDF support. Curve-fitting analysis of the XPS spectra for the laccase immobilized PVDF-PAA membranes fit well with the expected bond chemistry found in the enzyme (Figure 3d). The peak at the largest binding energy at 285 eV can be attributed to the abundant C-C bonds while the shoulder near 286 eV arises due to C-N bonding in the peptide backbone. The peak near 289 eV is a broad peak and can be attributed to both

peptide (N-C=O) and the carboxylate (O-C=O) bonds. The signature of C-F bond due to the PVDF support is still visible near 292 eV. These results further substantiate the successful installation of polymerized PAA and laccase into the PVDF membranes.⁴

Surface zeta potential analysis

After polymerization of PAA, the next step in preparing the membranes was to add PAH and laccase. The success of these steps was assessed via comparison of the membrane zeta potentials, as the zeta potential measures the surface charge on the membrane. Figure 4 shows the comparison of pH dependence of the zeta potential for PVDF membranes to that of functionalized PVDF membranes. The PAA-functionalized membrane shows an obvious phase transition near pH 3, which is attributable to the pKa of carboxyl groups in PAA. The surface charge for the PAH immobilized membranes were positive, as expected, due to the presence of the ammonium ions. The isoelectric point of the membranes shifted to ~3.5 after laccase immobilization (redline in Figure 4). These data are consistent with the literature value of ≈ 3.5 for the pI value of laccase from *Trametes versicolor*³⁸ suggesting successful immobilization of laccase onto the PAA functionalized PVDF membranes.

Water permeability studies and pH responsive flux of PVDF-PAA membranes

PAA hydrogel can be made to swell or shrink via tuning of its protonation state. At high pH it swells, producing contraction of the effective pore size. When the pH is decreased, the polymer shrinks and the pores are returned to their original volume. The significance of pH in the responsive behavior of PAA functionalized membranes is thoroughly documented in the literature and serves as a definitive test of PAA functionalization inside the membrane pores.^{39–40} Therefore we profiled the effect of pH on the water permeability of PVDF-PAA functionalized membranes (Figure 5). The flux of the membrane decreased with increasing pH consistent with successful polymerization of PAA within the PVDF pores, and previous findings.³³ Data in figure 5b were fit with the equation (1)⁴¹ yielding a pKa of 5.5, where $L_{p,max} = 193.4$ (high pH) and $L_{p,min} = 5.2$, where L_p is membrane water permeability, LMH/bar (low pH). It is worth mentioning here that with further immobilization of the layers of PAH and laccase (at respective pHs) onto the polymer matrix the flux dropped with time, signifying the successful immobilization of the components.

$$L_p = \left\{ L_{p,max}^{1/2} - \frac{[COO^-]}{[COOH] + [COO^-]} (L_{p,max}^{1/2} - L_{p,min}^{1/2}) \right\}^2 \quad \text{EQ 1}$$

Where,

$$\frac{[COO^-]}{[COOH] + [COO^-]} = \frac{1}{1 + 10^{(pKa - pH)}}$$

Catalytic activity of free and immobilized laccases

The kinetic parameters of enzymatic catalysis K_M (the Michaelis constant) and k_{cat} (the turnover number) for both free and immobilized laccase were determined using ABTS as the model substrate. The k_{cat} reflects the rate of the catalytic steps whereas the K_M signifies the substrate binding efficiency of the enzyme. The Table 2 data demonstrate that the apparent k_{cat} value of immobilized enzyme is comparable to that of the free laccase, indicating that immobilization via the LBL strategy does not impair the catalytic active site. The apparent Michaelis constant, K_M , for the immobilized membrane, however, increased nearly six times than the free enzyme. This higher K_M of immobilized laccase than that of free laccase is consistent with restriction of substrate access to the active sites of the immobilized enzymes by slowed diffusion through the polymer matrices combined with repulsion by the polyelectrolytes. Similar observation of elevated K_M values for immobilized systems compared to the free enzyme are reported in the literature, while the k_{cat} values for immobilized systems lies anywhere between comparable to lower depending on the methodology and the success of the immobilization method.⁴² This higher K_M for immobilized laccase compared to free laccase, decreased the catalytic efficiency (k_{cat}/K_M) of the enzyme in the functionalized membrane.

Stability of free and immobilized laccases

For industrial application of biocatalysts better storage stability is an essential criteria. Thus, the stabilities of solution phase laccase and membrane-immobilized laccase were examined, both stored at 4 °C, and the results are shown in Figure 6. The laccase immobilized membranes were stored immersed in DIUF water (pH 5.9). Solution phase laccase lost 30% of its initial activity after 3 days and 65% after 22 days of storage. However negligible loss of activity was detected for the membrane-immobilized laccase. These results are consistent with earlier reports on stability of immobilized laccase.³⁸ Enzymes are subject to damage mainly by other enzymes, chemicals, and extremes of pH and temperature. Unlike in the solution state, immobilization imparts extra stability to the enzyme as it prohibits the individual enzyme molecules from interacting with each other. In another words, the unfolding movements of the enzymes are restricted thereby inhibiting subsequent aggregation processes under these conditions.⁴³ Furthermore, as the immobilized membranes were stored immersed in DIUF water there is little chance of contamination with other chemicals. One more interesting advantage of the immobilization in membrane is that, the immobilized enzymes are inaccessible to bacteria due to the small size of the pores and hence to other enzymes that those bacteria might produce. Lastly, the storing pH is near neutral, so there are no concerns over the denaturation of the protein during storage. These factors combined together contribute towards the higher stability of the membrane immobilized enzyme compared to the solution phase.

Degradation and removal of TCP

In order to demonstrate the applicability of the laccase-functionalized membrane towards remediation of contaminated water, an aqueous solution of TCP was passed through the membrane under positive pressure of air from a gas tank. It should be noted here that laccase uses oxygen as the electron accepting secondary substrate during the oxidation of the

primary substrate. Thus the air flow from the air gas tank serves dual purpose; (i) ensures continuous oxygen atmosphere and (ii) maintains necessary positive pressure for controlling the flow rate. Initially the permeate was analyzed by UV-Visible spectroscopy to check the changes in the absorption behavior of TCP to confirm its conversion to metabolic products. TCP absorbed in a wide range from 280–300 nm (absorption maxima at ~295 nm) with two shoulders around 230 nm and 310 nm (please see supporting information, Figure S3). The permeate (light pink in color), however, absorbed at significantly different wavelength with a peak maxima at 277 nm, presumably attributable to the formation of dichloro-1,4-benzoquinone,⁴⁴ which clearly demonstrates the metabolism of the TCP molecules. A closer look at the spectra in the visible region displayed another broad peak near 525 nm which can be attributed to the formation of 2,6-dichloro-3-hydroxy-1,4-benzoquinone.⁴⁴ HPLC analysis along with the UV-vis spectra of the permeate solutions revealed diminution of the TCP content and new presence of various oxidation products of TCP (please see supporting information, Figure S4). Similar observations were made by Hu *et al.* in their study of degradation of TCP with the formation of various oxidation products along with a pink coloration.⁴⁴ LC-MS analysis demonstrated that the peak recorded at about 8 minutes corresponds to dichloro-1,4-benzoquinone, the major degraded product of TCP (please see supporting information, Figure S5). That the reaction corresponds to dechlorination was further confirmed by detection of approximately 1 mol chloride/mol TCP degraded, as determined using a chloride sensing electrode (Figure S6). Control experiments were performed to check the presence of enzyme leached with the permeate during the degradation of TCP. We neither saw significant amount of enzyme by Bradford assay (please see supporting information, Figure S2b) nor saw significant enzyme activity towards ABTS (0.2 mM, pH 5.8) in the aliquots from the permeate. It should be noted that had the enzyme been leached out from the membrane (and the degradation of TCP had been due to the released laccase in the permeate), there would have been a gradual change in the concentration of the TCP in the collected permeate samples over time. However, the amount of TCP and the chloride concentration in the permeate collected did not change with time (within ~24 hours) confirming no significant leaching of enzyme with the permeate.

Under our HPLC conditions, TCP had a retention time of 11 minutes (please see supporting information, Figure S4) and this peak was used to quantify the amount of TCP degraded as a function of different flow rates (Figure 7a). The data indicated that more than 80% of the TCP could be degraded at flow rate of 2 LMH under applied pressure of 0.7 bar or lower. This data showing remarkable degradation at a considerably low applied pressure, suggests an encouraging energy efficient flow through system with potential implementation beyond lab scale. The inset in Figure 7a shows the extent of TCP degradation as a function of residence time. It should be noted here that residence time is the average length of time during which a substrate is travelling through the pores and is different than a clock time. Residence time was calculated based on the permeability of the membranes. A high residence time indicates a lower flow rate which corresponds to lower applied pressure. Data in figure 7a demonstrate the advantage of flow through system over a batch reaction, degrading as high as 83% of the initial substrate in only 36 seconds residence time, which in a batch reaction takes hours. Table 3 summarizes several recent reports on both solution phase and immobilized laccase with applications mainly towards degradation of organic

pollutants in waste water.^{45–53} These reports, including this current study, agree on moderately good activity of the immobilized laccase to support its potential application in the wastewater management system. Despite the substantial amount of research on laccase immobilized systems the practical applicability of this versatile enzyme is still limited mainly due to stability issues and resistance towards harsh pH and chemicals. Use of membrane as the support material might be an answer to such issues as undesired chemicals can be restricted from coming into contact with the enzyme and secondly any destructive by product formed during the degradation can be washed away instantly.

In order to demonstrate the degradation activity of the membranes with variable loading of enzyme, a series of membranes with three different enzyme loading, viz. 3.2, 3.8, and 9.7 mg, were tested for its ability to degrade TCP. Normally as enzyme loading is tripled, one expects the conversion to go up by about 1.5 times if all the active sites can be accessed. Fig 7b shows the conversion did increase substantially (about 0.75 times) as the enzyme loading is tripled for a fixed residence time of 12 s. Figure S8 shows the overall effect of the enzyme loading on TCP conversion. This does indicate the impact of active sites crowding in the LBL membrane domain with high enzyme loading. Figure 7c shows the reusability of the enzyme-functionalized membrane as assessed as retention of capacity to catalyze degradation of TCP under convective flow mode. As evident from the figure, after 4 cycles of operation our enzyme-functionalized membrane displayed a loss of only 14%, of its initial activity, despite the duration of use of 25 days. As noted earlier, degradation of TCP leads to the formation of various oxidation products, some of which gets absorbed into the polymer matrix over time even after rigorous washing of the membranes. This limits the accessibility of the substrate TCP to the enzyme active site portraying a lower activity of the membrane during subsequent uses. As we do not see leaching of enzyme during the degradation studies some deactivation of the immobilized enzyme by the oxidized products seems to be a possible explanation of the loss of activity after repetitive use. We have also checked the reusability of the laccase functionalized membranes by regenerating the membranes by detaching the used laccase followed by immobilizing fresh laccase enzyme. The dislodging was achieved by permeating a 0.5 M NaCl solution (pH 7) through the membrane under pressure driven flow and then the fresh laccase was immobilized as mentioned before. ABTS activity assay of the dislodged membrane showed lowering of activity signifying detachment of approximately 85% of the immobilized laccase (data not shown). The regenerated membrane after re-immobilization of fresh laccase enzyme showed comparable activity to freshly prepared membrane as confirmed by the ABTS activity study. The detachment - reimmobilization steps were repeated to obtain consistent results. This study demonstrates the reusability of the PAA-PAH functionalized membrane domain for laccase enzyme immobilization through LBL approach, which is significantly advantageous over covalent modification technique.

CONCLUSIONS

Fabrication of PVDF microfiltration membranes as platforms for laccase immobilization is demonstrated. Laccase was successfully immobilized on the membrane using the established layer-by-layer assembly method. Although layer-by-layer assembly technique is known and used in various studies of enzyme immobilization, to our best of knowledge, this is the first

ever study of laccase immobilized membrane showing application under convective flow mode. The membrane-bound laccase displayed comparable activity to that of free solution phase laccase, as indicated by the Michaelis-Menten parameters. This is mainly because there is no physical bond formation or breaking involved other than short range/non-covalent interactions or ionic interactions. In this respect we anticipate the k_{cat} of the immobilized laccase to be comparable to that of free laccase. The higher apparent Michaelis constant, K_M , for the immobilized membrane can be accounted for by restricted access of substrate to the active sites of the immobilized enzymes by slowed diffusion. However, slight conformational changes of the enzyme during immobilization and possibility of hindrance offered by the polyelectrolytes to the substrate can't be ruled out. The laccase immobilized membrane was used to remove TCP, resulting in more than 80% degradation at a favorable flow rate of 2 LMH under a lower applied pressure of only 0.7 bar. This data suggests that unlike batch mode reaction, convective flow method can lead to degradation of ~80% of TCP in a residence time of only 36 s^{-1} . Combination of HPLC, LC-MS and UV-Visible spectroscopic analysis demonstrated dichloro-1,4-benzoquinone to be the major degraded product of TCP. The impressive performance of the laccase functionalized membrane along with the improved storage stability and the reusability for the oxidation of chloroorganics, indicate its potential for industrial applications in the field of wastewater bioremediation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LBL	Layer-by-Layer
PVDF	polyvinylidene difluoride
TCP	2,4,6-trichlorophenol
PAA	Polyacrylic acid
APS	ammonium persulfate
PAH	Poly(allylamine-hydrochloride)
MBA	N, N'-methylenebisacrylamide
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
LMH	Liter/m ² h
DIUF water	Deionized ultra-filtered water

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Figure 1.
ATR FT-IR spectra of PVDF and laccase-functionalized PVDF-membranes

Figure 2.

Scanning Electron Microscopy (SEM) images of (a) bare PVDF membrane, (b) PAA functionalized PVDF membrane and (c) Laccase Immobilized PVDF membrane.

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Figure 3.

XPS survey spectra of (a) bare PVDF membrane, (b) PAA functionalized PVDF membrane, XPS C1s core-level spectra of (c) PAA functionalized PVDF membrane and (d) laccase immobilized PVDF-PAA membrane.

Figure 4. Comparison of the Zeta Potential as a function of pH for PVDF, PVDF-PAA, PVDF-PAA-PAH and PVDF-PAA-PAH-laccase functionalized membranes.

Figure 5.

(a) pH-responsive flux and (b) effect of pH on the water permeability of PVDF-PAA membranes. Linear fits of the data in panel a yielded the permeability as the slope, which were replotted in panel b. PAA content = 14% wt. PVDF-PAA membrane area = 13.2 cm². T = 22 °C.

Figure 6. Comparison of storage stability of solution phase and membrane-immobilized laccase stored at 4 °C (DIUF water, pH 5.9).

(a)

(b)

(c)

Figure 7.

(a) Degradation of TCP by laccase-functionalized membrane as a function of flow rate 3.2 mg enzyme, (b) Degradation of TCP as a function of enzyme loading onto the laccase-functionalized membranes for a residence time of 12 s, and (c) Reusability of the laccase-functionalized membrane for residence time of 12 s. Experiments were performed at a temperature of 22 °C and a pH of 5.6. The initial substrate concentration was 250 μ M.

Scheme 1.

Schematic of laccase immobilization in membrane platforms through layer-by layer approach: PVDF-PAA membrane with an electrostatically immobilized layer of PAH and subsequent laccase immobilization. (In the scheme 'with respect to' is abbreviated as 'wrt').

Table 1

Contact angle measurements for the membranes after each stage of fabrication

Stages of Functionalization of Membrane	Contact Angle (°)
Bare PVDF Membrane	79.96 (± 3.86)
Functionalized with PAA	55.90 (± 0.06)
Functionalized with PAA and PAH	56.69 (± 2.24)
After Enzyme Immobilization	56.10 (± 1.08)

Table 2Michaelis-Menten kinetic parameters K_M and k_{cat} of free and immobilized Laccase

<i>Laccase</i>	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}\text{mM}^{-1}$)
Free	13.8 ± 1.9	21.1 ± 0.7	1528.9 ± 217.5
Immobilized	88 ± 5.3	22.7 ± 0.7	257.9 ± 17.6

Table 3

Several recent reports on laccase immobilization and their performance

Support	Application	pH	Degradation Performance (%)	Reference
Solution phase	Chlorophenol degradation	6	82.1	Reference 45
Solution phase	Chlorophenol degradation	5.6	83	This work
Wheat grains	Chlorophenol degradation	4.5	52.1	Reference 46
Silica	Chlorophenol degradation	5	86.9	Reference 47
Polyacrylonitrile Nanofibrous Membrane	Chlorophenol degradation	4.5	87	Reference 23
Oxidized Bacterial cellulose – TiO ₂	Dye degradation	5–6	80	Reference 49
MWCNTs modified LCEFM	Bisphenol A	4–7	80	Reference 48
Chitosan/CeO ₂ microspheres	Dye degradation	5.4	83.3–92.6	Reference 50
Mesoporous silica-modified electrospun	Dye degradation	-	88	Reference 52
Carbon-based mesoporous magnetic composites	Phenol and chlorophenol degradation	6	78–84	Reference 51
Magnetic Cu ²⁺ -chelated silica support	Chlorophenol degradation	5	82.9	Reference 53
PVDF Membrane	Chlorophenol degradation	5.6	60–83	This study