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Mild and Highly Flexible Enzyme-Catalyzed Modification of Poly(ethersulfone) Membranes

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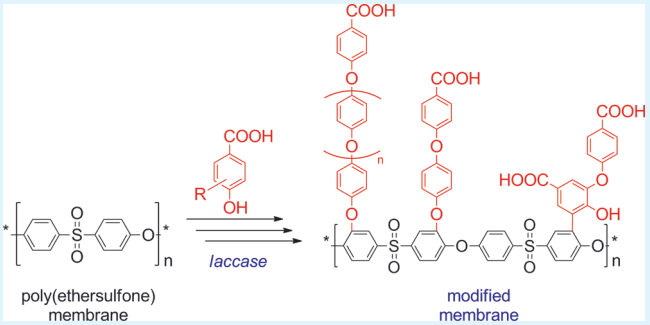
ABSTRACT: Poly(ethersulfone) (PES) membranes are widely used in industry for separation and purification purposes. However, the drawback of this type of membranes is fouling by proteins. For that reason, modification of PES membranes has been studied to enhance their protein repellence. This paper presents the first example of enzyme-catalyzed modification of PES membranes. Various phenolic acids (enzyme substrates) were bound to a membrane under very mild conditions (room temperature, water, nearly neutral pH) using only laccase from *Trametes versicolor* as catalyst. The extent of modification, monitored, for example, by the coloration of the modified membranes, can be tuned by adjusting the reaction conditions. The most significant results were obtained with 4-hydroxybenzoic acid and gallic acid as substrates. The presence of a covalently bound layer of 4-hydroxybenzoic acid on the grafted membranes was confirmed by X-ray photoelectron spectroscopy (XPS), infrared reflection absorption spectroscopy (IRRAS), and NMR. In the case of gallic acid, PES membrane modification is mainly caused by adsorption of enzymatically formed homopolymer. The ionization potential of the substrates, and the electronic energies and spin densities of the radicals that are intermediates in the attachment reaction were calculated (B3LYP/6-311G(d,p)) to determine the reactive sites and the order of reactivity of radical substrates to couple with the PES membrane. The calculated order of reactivity of the substrates is in line with the experimental observations. The calculated spin densities in the phenolic radicals are highest at the oxygen atom, which is in line with the formation of ether linkages as observed by IRRAS. The liquid fluxes of the modified membranes are hardly influenced by the grafted layers, in spite of the presence of a substantial and stable new layer, which opens a range of application possibilities for these modified membranes.



KEYWORDS: enzyme-catalyzed modification, surface modification, poly(ethersulfone) membrane, protein repellence, laccase, phenolic acid

INTRODUCTION

Poly(ethersulfone) (PES, see Figure 1) is a thermoplastic material that is abundantly used for polymeric ultrafiltration and microfiltration membranes, as its structural and chemical stability provides significant robustness. However, PES is a hydrophobic material with a relatively low surface energy and a correspondingly high water contact angle, and PES-based membranes are thus highly susceptible to adsorptive fouling. Therefore, various modification methods for PES membranes have been published that alter its surface properties, and ideally, do not influence its robustness.[1] Low-fouling PES membranes were obtained by blending of native PES with poly(vinylpyrrolidone), polyethylene glycol, and cellulose acetate phthalate,[2−4] and by coating with TiO_2 nanoparticles.[5] Photochemically initiated grafting methods[6−8] have been successfully applied to change PES membrane surface characteristics, and chemical modification of the structurally related polysulfone membranes has been achieved.[9−12] All these methods require either highly reactive reagents or high energy (photons, temperature) to achieve the required modification, as PES is a desirable material precisely because of its chemical inertness.

Enzymatic reactions are known for their mildness and eco-friendliness. As a result, enzymes are often applied as processing aids in food industries, where mild conditions are favored to avoid side reactions. Enzymes have also been used for the modification of polymers. Chitosan films have been successfully modified with a couple of phenolic compounds (e.g., hexyloxyphenol and 4-hydroxybenzoic acid) using tyrosinase.[13−15] Modification of membranes using enzyme-catalyzed grafting is potentially a very flexible technique to fine-tune membrane surface properties, but to the best of our knowledge, this has

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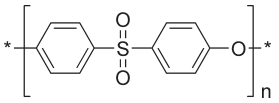

Figure 1. Molecular structure of PES.

not yet been applied to PES membranes, likely due to its limited chemical reactivity. For that reason, we decided to explore this technique, and present in this paper the mild and highly flexible modification of PES membranes with polar moieties using laccase from *Trametes versicolor*.

Laccase, initially obtained from the lacquer tree *Rhus vernicifera*,[16] and later from fungal sources mainly received attention within studies on enzymatic degradation of wood by white-rot fungi.[17,18] The physiological function of these biocatalysts is different in the various organisms, but they all catalyze polymerization or depolymerization processes of lignin or lignin-type building blocks, respectively.[16] In (bio)chemical terms, laccases [(EC 1.10.3.2)] are phenol oxidases. They catalyze the oxidation of a broad range of electron-rich substrates such as polyphenols, methoxy-substituted phenols, and aromatic or aliphatic diamines using a redox-active cluster of four copper ions as the active site.[17] The enzymatic product is the corresponding radical cation that rapidly loses a proton to give a reactive radical. The overall outcome of the catalytic cycle is the reduction of one molecule of oxygen to two molecules of water, which makes the enzyme environmentally friendly, and the concomitant oxidation of four substrate molecules to produce four radicals. Because, for example, phenolic radicals are highly reactive toward the phenol substrates, these intermediates can then produce dimers, oligomers, or polymers[16,18−21] that contain C−O or C−C linkages or both (see Figure 2 for an example of C−O linkages).[21]

The overall reaction rate depends both on the rate of the intermolecular electron transfer between catalytic Cu sites, and on the rate of product release.[22] Oxidations by laccase can be performed directly, i.e., the enzyme interacts with the administered substrate itself, or indirectly, in which case the enzyme oxidizes a chemical mediator that acts as an intermediate substrate. The oxidized radical of these mediators is subsequently able to react with, for example, the bulky substrates or with compounds having a high oxidation potential.[20] This stimulated us to precisely investigate PES-based materials, as the chemical inertness relates to the relatively electron-poor nature of the aromatic moieties.

Recently, laccase-mediated coating of lignocellulosic surfaces with polyphenols has been investigated, in order to obtain antibacterial performance.[23] The best conditions to make a laccase-induced coating were determined based on the obtained coloration and color depth of the formed layer, which depends on the nature of phenol (i.e., hydroquinone produces colorless, 2-methoxy-5-nitrophenol produces pale yellow, ferulic acid produces orange, and guaiacol produces a dark red layer). In a similar way, cellulose fibers were first functionalized by amine functional groups and subsequently coated with poly(catechol) "in situ" synthesized by *T. villosa* laccase.[24] In other applications, laccase-mediated grafting was used for dyeing of cellulosic fibers,[25,26] for attachment of anchoring groups for antifungals,[27] and phenol sulfonic acids,[28] or to improve the water resistance of wool fabric.[29]

The current paper presents a mild and highly flexible enzyme-catalyzed strategy for the modification of PES-based membranes, via the enzyme-mediated formation of phenolic radicals in the vicinity of PES membranes and subsequent covalent coupling of the radicals to the PES polymer through C−O linkages, or by strong adsorption of the formed homopolymers. This leads to modified PES membranes with more hydrophilic and tunable surface properties. Laccase was used under mild reaction conditions (water, room temperature, nearly neutral pH), and different phenol derivatives (see Figure 3) were used for the modification reaction. The phenol derivatives were chosen because of their expected reactivity toward laccase (laccase substrates), and because they contain a polar carbonyl group that will increase the hydrophilicity of PES. The modified membranes were analyzed by color measurement, X-ray photoelectron spectroscopy (XPS), infrared reflection absorption spectroscopy (IRRAS), and NMR. The ionization potential of the substrates, the energies of both the substrates and resulting radicals, and the spin densities in these radicals were calculated by quantum chemical means (B3LYP/6-311+G(d,p)//B3LYP/6-311G(d,p)), to further understand the modification reaction. Finally, membrane fluxes were checked to trace any negative influence of the modification method.

MATERIALS AND METHODS

Chemicals. Syringic acid (purity >98%), vanillic acid (98%), and 4-hydroxybenzoic acid (99%) were purchased from Alfa Aesar. Ferulic acid (>98%), catechol (>99%) and laccase from *Trametes versicolor* (22.4 $U \cdot mg^{-1}$) were obtained from Fluka. From Sigma-Aldrich were purchased: 2-fluoro-6-hydroxybenzoic acid (97%), 3-fluoro-4-hydroxybenzoic acid (95%), 4-fluoro-3-hydroxybenzoic acid (97%), gallic acid (>97.5%), sodium acetate (anhydrous, ≥99%), acetic acid (99.9%), and deuterium oxide (D_2O). 2,2′-Azobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Calbiochem. Flat sheet commercial poly(ethersulfone) membranes were purchased from Sartorius (symmetric, 0.2 μm pore size, 50 mm diameter, 150 μm thickness, water flow rate >28 $mL \cdot min^{-1} \cdot cm^{-2}$ at $\Delta P = 1$ bar). All chemicals were used as received. Milli-Q water was used in all experiments.

Laccase Assay. Laccase activity was determined with 2,2′-azobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The assay mixture contained 0.33 mL of 1 M ABTS solution, 2.67 mL of 0.1 M sodium acetate buffer (pH 5), with 0.05 U mL^{-1} laccase. Oxidation of ABTS is monitored by following the increase in absorbance at 420 nm (ε = 36 000 M^{-1} cm^{-1}).[30] The reaction time is taken as 1 min. Alternatively, the activity assay was carried out with catechol as substrate, using 0.33 mL of 10 mM catechol solution, 2.67 mL of 0.1 M sodium acetate buffer (pH 5), with 0.025 U mL^{-1} laccase. Oxidation of catechol is monitored by following the increase in absorbance at 400 nm (ε = 26 000 M^{-1} cm^{-1}),[31] with a reaction time of 20 min. One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 μmol of ABTS or catechol per min at 25 °C. The apparent kinetic parameters K_m and V_{max} were determined by fitting the initial reaction rate (v) and substrate concentration [for ABTS K_m = 258 mM and V_{max} = 455 × 10^{-3} mM min^{-1}, and for catechol K_m = 8.4 mM and V_{max} = 50.3 × 10^{-3} mM min^{-1}]. These values are comparable to the values found for fungal laccases.[32]

Color Measurements. The CIELAB coordinates for the modified membranes were measured with a ColorFlex (Hunter Lab, CIE L^*, a^*, b^*, CIE L^*, C^*, h^* at D 65/10°). The color values L^* (lightness), a^* (red-green axes), b^* (yellow-blue axes), and C^* were determined as average of three readings. Aperture size was 28 mm diameter. All parameters are determined relative to the unmodified membrane (i.e., ΔL^*, Δa^*, etc). ΔC^* (color saturation), is a characteristic parameter indicating the vibrancy or intensity of a color; a color with a high saturation will

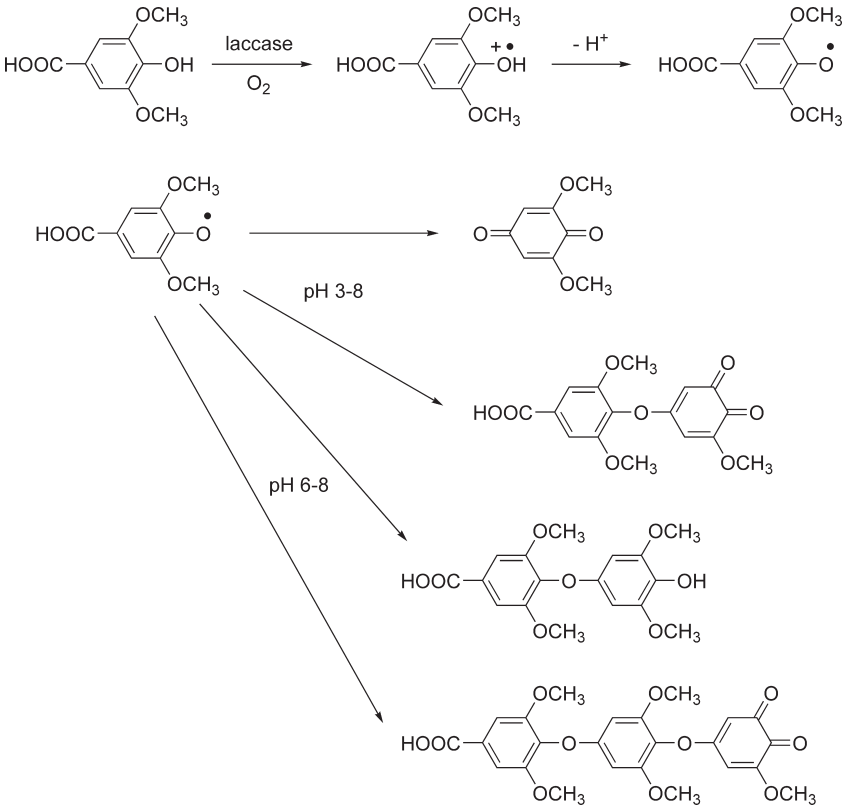

Figure 2. Transformation products from syringic acid (upper left) by laccase of *Rhizoctonia praticola* and *Trametes versicolor* at different pHs.[21]

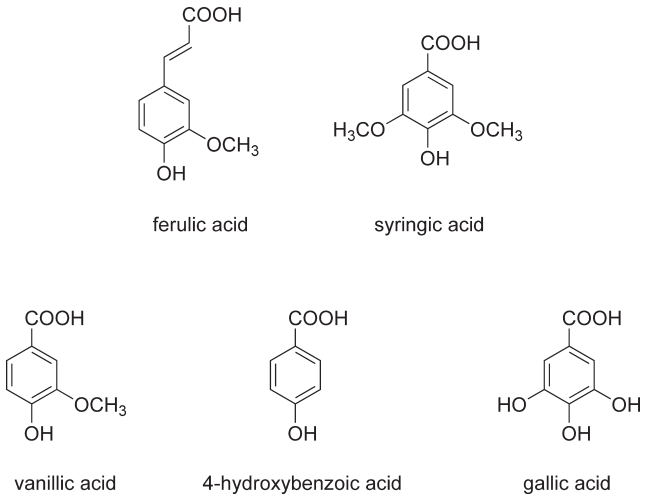

Figure 3. Phenolic monomers (substrates) used in this study.

appear more intense than the same color with less saturation. ΔE^* is the degree of total color change, which is calculated from $[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$.

X-ray Photoelectron Spectroscopy (XPS) Analysis. A JEOL JPS-9200 X-ray Photoelectron Spectrometer (Japan) is used for surface analysis of the elemental composition of the modified membranes to a depth of around 5 nm. High-resolution spectra were obtained under UHV conditions using monochromatic Al Kα X-ray radiation at 12 kV and 25 mA, using analyzer pass energy of 10 eV. All high-resolution spectra were corrected with a linear background before fitting.

Infrared Reflection−Absorption Spectroscopy (IRRAS) Analysis. The IRRAS spectra of phenolic acid grafted-membranes were obtained with a Bruker Tensor 27 FT-IR Spectrometer equipped with either an MCT-detector and the Auto-Seagull reflection module from Harricks, or a Bruker Hyperion 2000 FT-IR microscope. The Autoseagull is working with a single angle (range from 85° until 10°) while the Hyperion is equipped with a grazing IR-objective working with all angles in the range from 52 to 84°. The spectra of modified membranes are measured using the unmodified membrane as background. Spectra are expressed as % of reflectance.

Scanning Electron Microscope (SEM). Scanning of membrane cross-sections were carried out using a Magellan 400 SEM (FEI, Eindhoven, The Netherlands). The samples were prepared by fracturing in liquid nitrogen and followed by coating with Pt. The applied voltage was 3 KV; the resolution was 1024 × 884 pixels.

Pure Water Flux and Membrane Hydraulic Resistance (R_m). A dead-end stirred filtration cell (Millipore, model 8050, active transport area 13.4 cm^2) was used to characterize the filtration performance of unmodified and modified membranes. Pure water flux was measured at a constant trans-membrane pressure of 1 bar at 24 ± 1 °C and 300 rpm. The pure water flux is calculated with eq 1, in which Q = quantity of permeate collected (m^3), J_w = water flux ($m^3\ m^{-2}\ s^{-1}$), Δt = sampling time (s), and A = the membrane area (m^2).

$$Q = \frac{J_w}{\Delta t A} \quad (1)$$

To determine the hydraulic resistance of the membrane (R_m), the pure water flux was measured at different transmembrane pressures of 0.2, 0.4, 0.6, 0.8, 1, and 1.2 bar, respectively. The resistance of the membrane follows from the slope of water flux versus transmembrane pressure.[33]

Membrane Modification Experiments. For the basic screening experiments, flat membranes were incubated in 40 mL 0.1 M sodium acetate buffer (pH 5) containing different concentrations of phenolic acids (monomers) and enzyme. The samples were gently shaken, and after a specific incubation time, they were removed from the liquid. Ferulic acid, vanillic acid, syringic acid, 4-hydroxybenzoic acid, and gallic

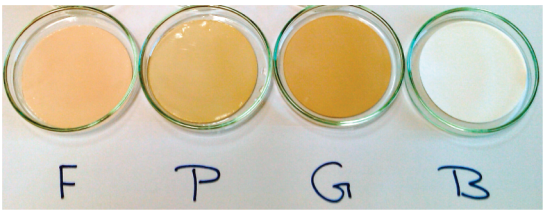

Figure 4. Color changes of modified circular membranes (wet membranes) after enzyme-catalyzed modification with ferulic acid (F), 4-hydroxybenzoic acid (P), gallic acid (G), (B) is the blank membrane.

acid were tested, all at concentrations of 0.6, 10, and 28.8 mM, respectively, and with modification times of 0.5, 2, 8, and 24 h. The enzyme concentration tested was 0.5 U·ml^{-1}. After the modification time was completed, the membranes were washed by strong flushing followed by repeated dipping in Milli-Q water. The modified membranes were kept in glass-covered dishes in desiccators supplied with silica gel for drying. Additional experiments were done with fluorohydroxybenzoic acids, to prove their coupling to the membrane by XPS. The resulting membranes were treated in the same way as described before.

Grafting Yield. The amount of phenolic acid grafted onto the membrane surface is calculated from the weight of the membrane, before and after grafting, and the grafting yield is expressed as the weight increase relative to the initial weight. Before grafting, all the membranes were kept for 24 h in glass-covered dishes in desiccators supplied with silica gel to remove any moisture.

NMR Studies. ^{1}H NMR spectra were recorded on a Bruker AVANCE III NMR resonating at 400 MHz, equipped with an inverse broadband gradient probe. A part of a membrane disk modified with 4-hydroxybenzoic acid (substrate concentration 28.8 mM, enzyme concentration 0.5 U/mL, sodium acetate buffer pH 5.0, 25 °C, modification time 24 h) was completely dissolved in DMF-d_7 and subjected to NMR analysis.

Blank Experiments. *Enzyme and Substrate Adsorption Test.* The unmodified membrane sample was incubated in 40 mL 0.1 M sodium acetate buffer (pH 5, 24 ± 1 °C) containing 1 U mL^{-1} enzyme in the absence of phenolic acid substrate, for 24 h. The amount of adsorbed enzyme was calculated from the weight difference. The same test was performed with the monomer solution (without enzyme).

Homopolymer Adsorption Tests. The reaction of phenolic acid substrate and enzyme was carried out as described above, but now without the PES membranes. The reaction time was 24 h for both 4-hydroxybenzoic acid and gallic acid. The enzyme was inactivated by adding 5 mL of 0.1 M NaOH while stirring for 10 min. After readjusting the pH to 5 with concentrated HCl, the PES membranes were incubated for 2 h in this solution. After the incubation time was completed, the membranes were washed by strong flushing followed by repeated dipping in with Milli-Q water. The membranes were kept in glass-covered dishes in desiccators supplied with silica gel for drying before color measurements.

Molecular Modeling. The geometries of radical substrates were optimized by B3LYP density functional theory calculations with the basis set (B3LYP/6-311G(d,p)) using the Gaussian03 program.[34] Frequency calculations were performed to ensure that the optimized geometry is a minimum on the potential energy surface. Single-point calculations (B3LYP/6-311+G(d,p)//B3LYP/6-311G(d,p)) were performed to obtain energies and spin densities, using the IOP settings IOP(5/14 = 2,6/26 = 4) in the input file section; the resulting projected $\langle S^2 \rangle$ values were below 0.751 in all cases.

RESULTS AND DISCUSSION

Incubation of the membrane with laccase and the substrates leads to visual changes of the membrane, as is illustrated in Figure 4; depending on the substrate used, the color is more or less intense. The reaction conditions were 28.8 mM substrate, 0.5 U·ml^{-1} enzyme concentration, 24 h modification time, 24 ± 1 °C, pH 5 (0.1 M sodium acetate buffer); these conditions are used throughout the paper unless noted otherwise. The aqueous medium solutions also colored with time (not shown) and this is attributed to the formation of homopolymers, due to reaction of enzymatically formed phenolic radicals with phenol monomers.

The experimental color changes show that reactions occur with the membrane, especially when ferulic acid, 4-hydroxybenzoic acid, and gallic acid are used. Moreover, it is found that the reaction rate (as qualitatively deduced from the rate of color change) is highest for gallic acid, followed by ferulic acid and 4-hydroxybenzoic acid. For vanillic acid, a reaction occurred albeit very slowly and at long modification time (a change in color is noticeable after 48 h modification time) and high monomer concentration. There was no notable color change with syringic acid even at high monomer and enzyme concentration, or prolonged modification time.

As control experiments, it was shown that no notable reaction occurred between PES membrane and any of the tested monomers in the absence of enzyme (no color change, no weight change), even after 24 h modification time. Incubation of the membrane with enzyme in the absence of substrate yielded some adsorption of enzyme on/in the PES membrane (142 mg·m^{-2} PES), but this did not lead to coloration or any other change of the membrane.

Because the colored membrane remains colored even after prolonged washing, covalent modification of the membrane is likely (see below for investigations regarding this claim). Presumably, the enzymatically generated radicals form covalent bonds to the membrane material, yielding a surface-bound radical that can subsequently undergo further reactions to form a surface-bound conjugated polymer. The resulting extended π-system gives rise to the observed color change.

As mentioned, gallic acid, 4-hydroxybenzoic acid, and ferulic acid show high reactivity toward the PES membrane. Therefore, our investigations initially focused on these three compounds, in order to establish a proof of principle for laccase-catalyzed modification of PES membranes under mild modification conditions. The amount of attached material was first evaluated from color development as a function of time and substrate concentration, as this turned out to be a quick method to evaluate a large number of samples. From the CIELAB analysis, lightness (ΔL^*), color saturation (ΔC^*), and degree of total color change (ΔE^*) were found indicative for the degree of modification. Obviously, the lowest ΔE^* value (greatest ΔL^*, Table 1) was obtained for the unmodified membrane. For membranes treated with ferulic acid, the ΔE^* value increased with increasing modification time, but not with increasing substrate concentration, thereby indicating that the used concentrations are in the saturation range of the enzyme. It is assumed that the enzymatic reaction is the rate-limiting step (i.e., the subsequent chemical reactions of the enzymatically formed radicals are much faster than the enzymatic oxidation). The lower coloration at high concentration and longer modification time is possibly due to enzyme inhibition and/or poor aqueous solubility of the ferulic acid under the used reaction condition. Because of these uncertainties we decided to continue our studies with just 4-hydroxybenzoic acid and gallic acid.

Membranes modified with 4-hydroxybenzoic acid showed an increase in ΔE^* value, both with increasing substrate concentration and modification time, indicating that the substrate concentrations

Table 1. Lightness, Degree of Total Color Change, and Color Saturation Response of Modified PES Membranes As Determined by CIELAB Measurements[a]

reaction conditions					
substrate	substrate concentration (mM)	modification time (h)	ΔL^*	ΔE^*	ΔC^*
unmodified membrane			100.0	0.0	0.0
ferulic acid	0.6	8	97.1	8.9	6.7
		24	93.9	14.0	10.7
	10	8	96.6	9.2	6.7
		24	94.3	11.4	7.9
4-hydroxybenzoic acid	0.6	8	99.2	4.3	2.5
		24	97.3	6.7	4.2
	10	8	96.1	7.9	4.8
		24	94.2	10.6	6.8
homopolymer adsorption blank test		24	96.3	7.3	4.7
gallic acid	0.6	8	98.8	3.0	0.8
		24	98.1	3.2	0.9
	10	8	96.4	4.8	1.3
		24	94.3	8.9	5.0
homopolymer adsorption blank test		24	94.7	7.8	3.8

[a] Typical error limits: ± 0.1 for lightness (ΔL^*) and color saturation (ΔC^*); ±0.2 for the degree of total color change (ΔE^*).

Table 2. Analysis of XPS Spectra of Modified PES Membranes

	C_{1s}	C_{1s}	O_{1s}	N_{1s}	S_{2p}
binding energy (eV)	285.4 ± 0.3	288.8 ± 0.5	533.2 ± 0.3	400.1 ± 0.1	169.0 ± 0.3
	C—C	C═O	—C—O—	—N—	O═S═O
sample			atomic %		
unmodified PES	77.01	16.99	15.56	2.15	5.30
4-hydroxybenzoic acid	76.18	9.30	19.79	1.98	2.05
gallic acid	74.95	3.62	18.38	2.10	4.65
gallic acid[a]	74.50	5.09	19.20	2.44	3.85

[a] 2 h modification time.

were below the saturation concentration of the enzyme. More polymer attachment is thus expected at higher substrate concentrations. Membranes could also be modified with gallic acid, as is evident from the color change. Free radical formation with gallic acid is extremely fast; a color change in the reaction solution is already apparent after a few seconds. At low gallic acid concentration, the difference in color change with increase of modification time is less; apparently, all gallic acid has reacted already. An increase in the gallic acid concentration allows the reaction to proceed for a longer time, resulting in noticeable color change with longer modification times. These observations are in line with the changes in both ΔL^* and ΔC^* values (see Table 1); it should be kept in mind, that an increase in ΔE^* corresponds to a decrease in ΔL^* and an increase in ΔC^*.

The observed color change in the membrane can be caused by covalent modification and/or by physical adsorption of substrate homopolymer, formed in solution, to the membrane. In order to study the latter possibility, enzymatic reactions were conducted in the absence of the PES membrane. After 24 h of reaction, membranes were immersed in the colored solution for 2 h, and the membrane color was determined, after extensive washing. Indeed, some coloration was found (see Table 1), indicating that homopolymer adsorption does occur and apparently yields strong noncovalent bonds to the membrane. For gallic acid it cannot be excluded that only physisorption accounts for the resulting surface modification, as incubation with enzyme and membrane yields the same coloration as polymerization followed by membrane immersion. The data for 4-hydroxybenzoic acid indicate that covalent attachment onto the PES membrane also takes place, as physisorption by itself cannot yield the same degree of coloration as observed from polymerization that takes place in the presence of PES membranes.

To study the attached layer on the PES surface, we carried out XPS analysis. In Table 2, the results for the unmodified and two modified membranes are shown. Most notable is the decrease in sulfur content, as observed in the intensity of the S_{2p} peak at 169.0 eV ($-\underline{S}O_2-$). This decrease is a good indication for the formation of an overlayer on the membrane that shields the underlying sulfur. For both carbon and oxygen, concentrations are found that are notably different from the unmodified membrane, which is another indication for the formation of an extra layer. These effects are pronounced for both phenolic acids; and the XPS results are, in general, in good agreement with our initial observations on color change. The presence of the nitrogen and the C═O peaks in the unmodified membrane are most probably due to incomplete leaching out of used solvent during

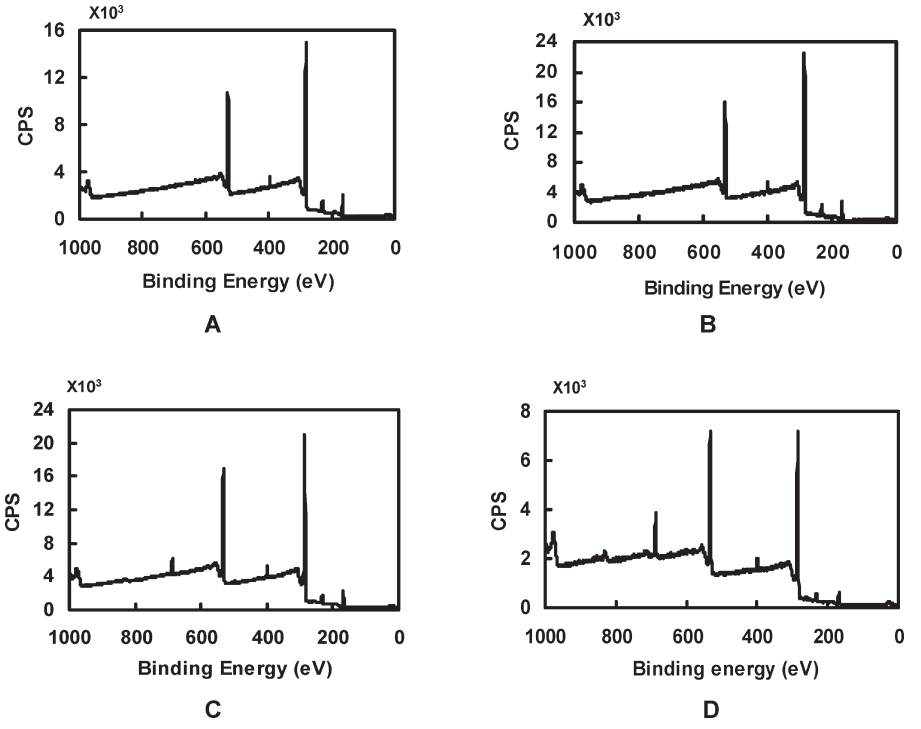

Figure 5. XPS spectra of unmodified and modified PES membranes with fluorinated hydroxybenzoic acids. (A) Unmodified membrane; (B) 2-fluoro-6-hydroxybenzoic acid; (C) 4-fluoro-3-hydroxybenzoic acid; (D) 3-fluoro-4-hydroxybenzoic acid.

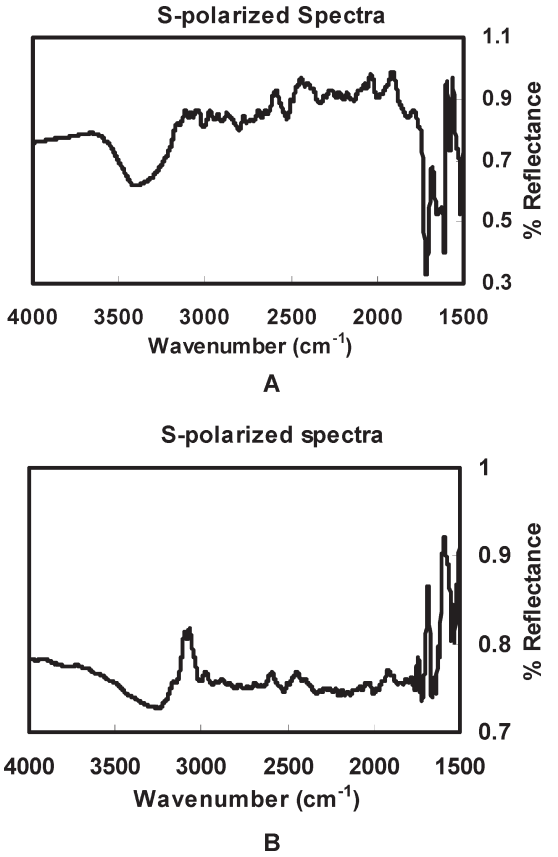

Figure 6. IRRAS spectra for (A) 4-hydroxybenzoic acid and (B) gallic acid modified membranes. Reflectance is relative to that of the unmodified membrane.

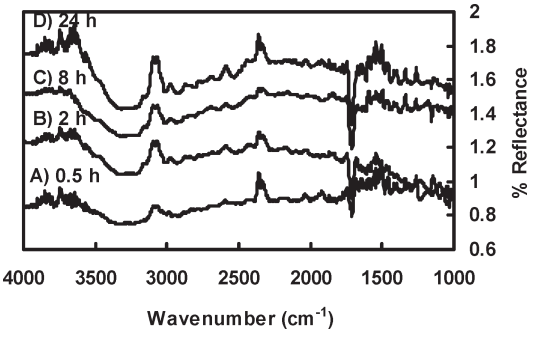

Figure 7. IRRAS spectra for 4-hydroxybenzoic acid-grafted membranes at (A) 0.5, (B) 2, (C) 8, and (D) 24 h modification time. Reflectance is relative to an unmodified membrane.

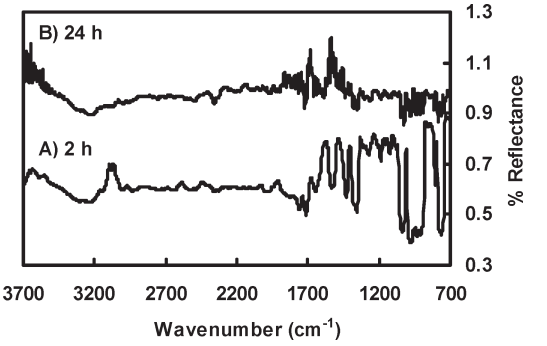

Figure 8. IRRAS spectra for gallic acid-grafted membranes at both (A) 2 and (B) 24 h modification time. Reflectance is relative to an unmodified membrane.

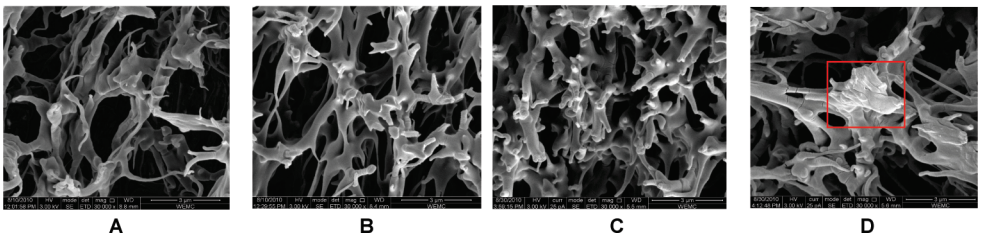

Figure 9. SEM images (30 000×) of cross-sections of (A) blank membrane, (B) 4-hydroxybenzoic acid (24 h modification time), and (C, D) gallic acid (2 and 24 h modification time, respectively).

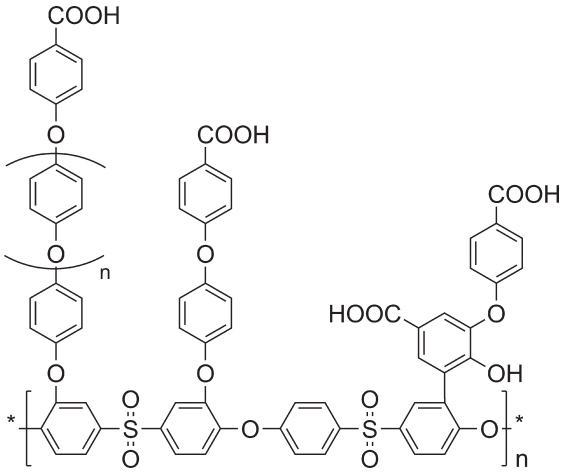

Figure 10. Schematic representation of a possible chemical structure of the PES surface after modification with 4-hydroxybenzoic acid, containing mainly O-linked structures with some C-linked compounds.

the phase-inversion fabrication of the PES membrane or due to presence of other used additive materials like polyvinylpyrrolidone. One would expect an increase in the C═O peak upon the covalent coupling of substrate molecules, but apparently decarboxylation takes place (see Figure 2 for an example from literature).

To further quantify the extent of the modification reaction, we used fluorohydroxybenzoic acids, as these allow for top layer-specific XPS analysis. Figure 5 shows the XPS signals for four different membranes: before modification (A), and after modification with 2-fluoro-6-hydroxybenzoic acid (B), 4-fluoro-3-hydroxybenzoic acid (C), and 3-fluoro-4-hydroxybenzoic acid (D). It was found that 2-fluoro-6-hydroxybenzoic acid does not react with the membrane under these conditions (no F_{1s} peak visible at 687 eV). Both 4-fluoro-3-hydroxy- and 3-fluoro-4-hydroxybenzoic acid react with the membrane surface but at different rates, resulting in 1.48 and 3.62% F, respectively, which is qualitatively in line with the overlayer grafted substrate (grafting yield): 1.32 ± 0.02, and 1.87 ± 0.02 g m^{-2} PES, respectively, for the two reacting substrates. Also, the lightness and the degree of color change of the samples were in agreement with these findings (ΔL^* = 96.8 ± 0.1 and ΔE^* = 7.3 ± 0.2 for 4-fluoro-3-hydroxybenzoic acid and ΔL^* = 94.6 ± 0.1 and ΔE^* = 9.0 ± 0.2 for 3-fluoro-4-hydroxybenzoic acid).

IRRAS spectra also confirmed that 4-hydroxybenzoic acid and gallic acid were bound to the surface as grafted oligomers and/or adsorbed polymers. At 3400−3500 cm^{-1} an OH peak was present in the modified membranes, as shown in Figure 6.

The IR-GIR spectra of the 4-hydroxybenzoic acid grafted membranes obtained after different modification times (0.5, 2, 8, and 24 h) are shown in Figure 7. A clear sharp C═O group peak at 1708 cm^{-1} indicates the presence of carboxylic acid. The intensity of this peak increases with increasing modification time, i.e., increased amount of grafting. The characteristic bands due to aromatic C−H stretch around 3030−3080 cm^{-1} (benzene ring) and the intense, broad band of OH around 3200−3500 cm^{-1} all refer to the newly attached phenolic compounds. The increase in modification time produces stronger signals, because of increased grafted amounts of 4-hydroxybenzoic acid. For gallic acid, two different modification times (2 and 24 h, see Figure 8) were used; the characteristic bands are more clear at lower modification time (2 h). This might be the result of the reaction of the remaining OH-groups with PES and other gallic acid molecules inside the modified membrane during prolonged incubation (i.e., gallic acid reacts much faster than 4-hydroxybenzoic acid).

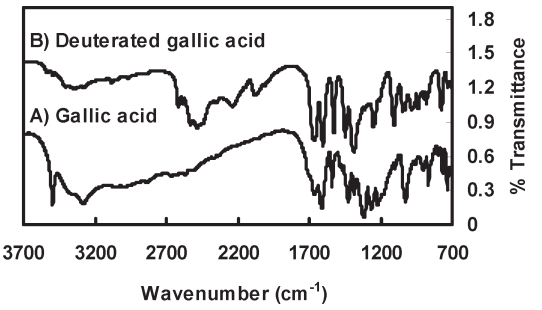

Figure 11. KBr-IR spectra for (A) gallic acid and (B) deuterated gallic acid, as KBR pellets.

All the used analysis techniques illustrate that both 4-hydroxybenzoic acid and gallic acid are efficiently bound to the membrane. This binding leads to a homogeneous and well-defined modification of the membranes, as is also borne out by scanning electron microscopy (SEM) images of membrane cross sections (Figure 9A−C). Only upon extended modification times for gallic acid (e.g., 24 h; Figure 9D), larger structures that appear in an inhomogeneous fashion (red rectangle in Figure 9D) start to appear, pointing to the necessity to limit the incubation time in this case to increase sample-to-sample reproducibility.

Linkage Type Exploration. The phenolic radicals produced by laccase can be O-centered or C-centered. Products derived from both species have been found in literature.[35−38] Generally, it can be expected that the radicals will react with carbon atoms of PES which have the highest electron density (i.e., the carbon atoms ortho to the ether linkages). In addition, the substrate can be bound to the PES as monomers or as oligomers as shown in Figure 10 for 4-hydroxybenzoic acid. The strong coloration indicates an extended conjugated system, which would point to oligomeric structures that link at least several benzene rings together.

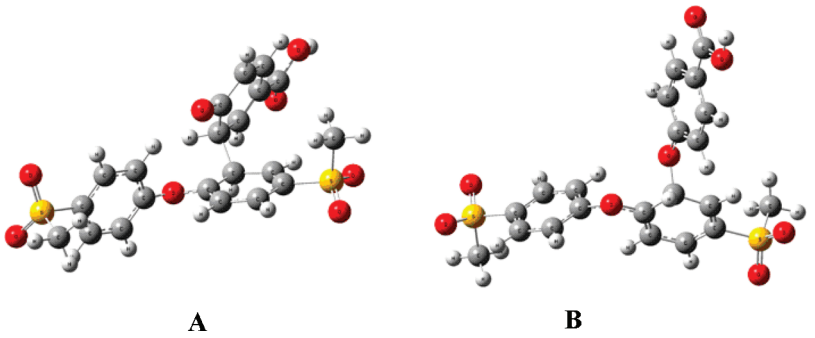

Figure 12. 4-Hydroxybenzoic acid coupled to PES polymer through (A) an aromatic carbon atom and (B) a phenolic oxygen atom.

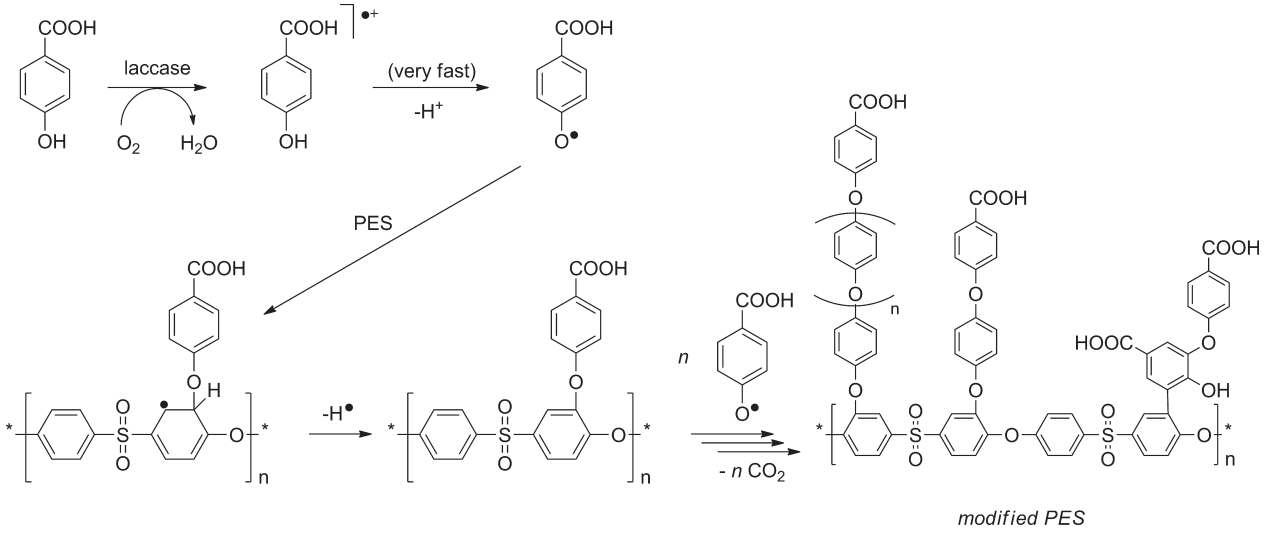

Figure 13. Tentative mechanism for the formation of reactive 4-hydroxybenzoic acid radicals by laccase and grafting of the radicals to PES membranes.

To explore if the polymerization of gallic acid predominantly takes place through C−O or C−C linkages, IR spectroscopy was carried out. Gallic acid shows peaks for O−H stretching and bending at 3463 and 1103 cm^{-1}, respectively, as proven by deuterium exchange (i.e., gallic acid was dissolved in deuterium oxide (D_2O) and freeze-dried to exchange the H by D atom, see Figure 11).[39] Upon binding to PES, these peaks are still visible after 2 h modification time but diminish after 24 h modification time (see Figure 8). This is a strong indication that gallic acid is enzymatically polymerized and bound to PES predominantly via O-linked oligomers or polymers.

In another study, the membrane modified with 4-hydroxybenzoic acid was dissolved in DMF-d_7 and subjected to ^{1}H NMR analysis. Clear signals of the ortho and meta protons of the grafted substrate were observed, but careful analysis showed that they were slightly shifted toward higher field compared to free 4-hydroxybenzoic acid. Spiking of the membrane solution with a small amount of 4-hydroxybenzoic acid clearly showed two sets of peaks: δ 7.922 and 6.953 (J = 8.66 Hz) for the membrane-bound 4-hydroxybenzoic acid, δ 7.927 and 6.957 (J = 8.61 Hz) for the free 4-hydroxybenzoic acid. Apparently the signals are derived from terminal O-linked 4-hydroxybenzoic acid units on the membrane.

These results are strong indications for O-linked coupling with a small fraction of C−C bond formation to account for the coloration, leading to the mixed structure shown in Figure 10.

Also the grafting yield indicate that the new layer on the membrane consists of oligomers or polymers rather than a monolayer. Assuming that one molecule (mass: 1×10^{-17} mg) occupies about 1 nm^2 of space, a dense monolayer would thus approximately yield a coverage of 1×10^{-17} mg/nm^2 = 10 mg/m^2. The obtained coverage of PES membranes by, for example, hydroxybenzoic acid is in the range of hundreds of mg/m^2, and thus is on average significantly more than a monolayer.

Molecular Modeling. Quantum chemical calculations were used to calculate electronic energies, spin densities, and ionization potential of the radical substrates used. Thereby, the order of reactivity of radical substrates, which coupled with the PES membrane, was determined. The experimental results showed that the modification process with gallic acid is faster than with 4-hydroxybenzoic acid (356.5 vs 101.8 mg m^{-2}; 4.8 mM substrate and 2 h modification time), and this is in good agreement with the lower ionization potentials obtained in the calculations (Table 3). Analysis of spin populations shows that there is a high spin density on the phenolic oxygen atom compared to other atomic sites of the two substrates involved in the PES modification reaction. In addition, the stability data for, for example, gallic acid mean that removal of the H on the −OH para to the COOH yields a radical that is 6.7 kcal/mol more stable than the radical obtained by removal of the meta −OH hydrogen atom.

To obtain additional evidence about the reactivity of substrates with respect to the PES membrane, the ionization potentials of the substrates were approximated by calculating the negative of their HOMO energies (Koopmans' theorem). As the one-electron oxidation is likely rate limiting in aqueous

Table 3. Ionization Potentials (IP) of the Substrates and −O· Spin Densities of the Resulting Phenolic Radicals (B3LYP/6-311+G(d,p)//B3LYP/6-311G(d,p) level of theory)

radical substrates	IP (eV)	spin density on −O·
4-hydroxybenzoic acid	7.09	0.333
gallic acid (para to COOH group)	6.63	0.280
gallic acid (meta to COOH group)	6.74	0.327

media, this provides an easily calculable reactivity descriptor. Gallic acid is more reactive than 4-hydroxybenzoic acid, as confirmed by the lower ionization potential; the near-equal spin densities point to only a minor influence of that factor in determining the relative reactivity. In order to get more insight into the stability of the PES-coupled substrate, the relative electronic energy of 4-hydroxybenzoic acid attached to PES (one unit) either through a phenolic oxygen atom or through an aromatic carbon atom (ortho to the $-O^{\bullet}$ moiety) was calculated. It is found that the oxygen-coupled product is more stable (10.4 kcal/mol) than the carbon-coupled one. (See Figure 12 for the optimized structures of both PES-coupled substrates.) Because it is likely that this difference is reflected in the relative transition state energies, albeit to a smaller degree, the latter energy difference serves as additional evidence for a PES grafting/polymerization mechanism predominantly but likely not exclusively via C−O bond formation. This theoretically predicted order of reactivity of substrates on the PES membrane is thus in line with the experimental observations, and reported polymerization products for both syringic and vanillic acid.[21] The overall reaction scheme for 4-hydroxybenzoic acid is presented in Figure 13.

Outlook for Laccase-Catalyzed Membrane Modification. We have shown that reactive radicals produced by laccase can be grafted onto the surface of PES membranes by formation of a covalent C−O linkage (see Figure 13). Because of the presence of many phenolic radicals in the reaction medium, extra monomers can be oxidatively grafted to the first attached monomer, with concurrent elimination of both hydrogen and carbon dioxide to form oligomers or polymers, as described elsewhere.[40] At the same time, these extra monomers can also be oxidatively grafted to other monomers or preformed oligomers to form homopolymers that can be partially adsorbed to the membrane surface. The latter reaction route is especially pronounced for gallic acid. Water is the only byproduct in this enzymatic reaction.

Interestingly enough, the flux of grafted membranes is not significantly influenced. At 1 bar applied pressure, the 4-hydroxybenzoic acid-modified membrane [28.8 mM substrate, 0.5 $U \cdot ml^{-1}$ enzyme, 0.5 h modification time, 24 ± 1 °C, and pH 5 (0.1 M sodium acetate buffer)] has a flux comparable to the unmodified membrane [25.9 ± 0.5 and 24.5 ± 0.5 mL cm^{-2} min^{-1} for unmodified and modified membrane, respectively]. Therefore, we expect, that through medium engineering, (i.e., substrate concentration, enzyme concentration, reaction pH, temperature, etc.) the enzyme-catalyzed modification technique for PES membranes can be tailored to serve various separation purposes.

CONCLUSIONS

Laccase-catalyzed grafting of PES membranes can be carried out using a variety of phenolic acids as substrates. For 4-hydroxybenzoic acid, it was shown that this compound is directly coupled to the PES, and that substantial amounts can be grafted to the PES membrane through O-centered coupling. For gallic acid, membrane modification mostly occurs via polymerization and strong adsorption of the homopolymer to the membrane. Interestingly, the fluxes of the modified membranes remained high (i.e., comparable to the unmodified membrane).

From our findings can be concluded that any so-called "chemically inert" membrane that contains PES or a related polymer such as polysulfone can be modified enzymatically under the mildest possible conditions (water, room temperature, pH 5), although clearly, further optimization is needed to "fine-tune" the modification layer to the separation for which it needs to be designed.

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