

Article

## Enzymatic Modification of Polyethersulfone Membranes

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**Abstract:** Enzymatic modification of polyethersulfone (PES) membranes has been found not only feasible, but also an environmentally attractive way to vary surface properties systematically. In this paper, we summarize the effect of modification layers on protein adsorption and bacterial adhesion on PES membranes and surfaces. The enzyme laccase was used to covalently bind (poly)phenolic acids to the membrane, and compared to other membrane modification methods, this method is very mild and did not influence the mechanical strength negatively. Depending on the conditions used during modification, the modification layers were capable of influencing interactions with typical fouling species, such as protein, and to influence attachment of microorganisms. We also show that the modification method can be successfully applied to hollow fiber membranes; and depending on the pore size of the base membrane, proteins were partially rejected by the membrane. In conclusion, we have shown that enzymatic membrane modification is a versatile and economically attractive method that can be used to influence various interactions that normally lead to surface contamination, pore blocking, and considerable flux loss in membranes.

**Keywords:** membrane modification; polyethersulfone; enzyme; laccase; phenolic acids; brush structure; protein adsorption; bacterial adhesion

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## 1. Introduction

Process and membrane development are considered equally important for successful operation of many membrane filtration processes. In this paper, we specifically focus on the use of polyethersulfone (PES) membranes; the high glass transition temperature of the material makes the membranes sturdy, allowing for steam sterilization. In addition, the material is known for its chemical stability. Unfortunately, PES is hydrophobic, which enhances interaction with many foulants. To influence this, PES is often blended with polyvinylpyrrolidone (PVP), which also allows for a wider variation in membrane specifications [1,2]. Still, PES membranes show a high binding affinity for proteins and microorganisms, which causes severe fouling of membranes during operation.

To diminish fouling, various modification methods have been suggested in the literature. For example, coating, blending, composite, chemical, grafting, or a combination of these methods, have been discussed in our recent review paper [3]. Membrane modification has become an important way of preventing undesired interactions with components in the feed, and although success stories can be found on either of these methods in literature, there is a lot of trial and error involved in arriving at an appropriate membrane formulation. In this respect, our recently published enzymatic modification method for polyethersulfone membranes is rather different [4], since it starts with chemically stable base membranes that are modified with the enzyme laccase that covalently attaches monomers, dimers, and oligomers to the surface without influencing the mechanical strength (which can be a great issue during classic membrane modification). Also, the flux of microfiltration membranes does not change too much (typically <10%), while still having a pronounced effect on interactions with foulants [5,6].

The enzyme laccase that is used in our method produces free radicals from suitable substrates (e.g., phenolic acids), using the oxygen from air as an oxidant and producing water as the only by-product. In the ensuing secondary reactions, monomers are attached to PES surfaces forming either a network, or more brush-type structures, depending on the substrate and reaction conditions used. Laccase has good thermal stability, does not suffer from substrate inhibition, and has high oxidation rates compared to other enzymes (10–100-fold higher than lignin peroxidase or manganese peroxidase) [7,8].

In this paper, we present the enzymatic modification method carried out with an enzyme from *Trametes versicolor*, together with results obtained with 4-hydroxybenzoic acid as substrate for the enzyme. The tentative reaction mechanism is shown in the supporting material leading to covalent bonds with the structure [4]; typical brush-type structures are formed, as illustrated there. (Under the reaction conditions used here, nonspecific binding of homopolymer is minimal.) We will show the effect of the modification layer on protein repellence in hollow fiber membranes, with those conditions that work best to investigate attachment of microorganisms. In the last section, we conclude with a short exposé on the costs related to enzymatic modification of PES membranes.

## 2. Experimental Section

### 2.1. Chemicals

Sodium acetate (anhydrous,  $\geq 99\%$ ), acetic acid (99.9%), 4-hydroxybenzoic acid (99%), potassium chloride (99%) and laccase from *Trametes versicolor* ( $>20$  U  $\text{mg}^{-1}$ ) were purchased from Sigma-Aldrich. Polyethersulfone (PES) (Ultrason, E6020P) was obtained from BASF (Ludwigshafen, Germany). Prime grade 150 mm silicon wafers of type P/B <100> orientation, thickness 660–700  $\mu\text{m}$ , and 2.5 nm native oxide layer were purchased from Wafer Net Inc. (San Jose, CA, USA). Brain heart infusion (BHI) broth was from Becton Dickinson, Le Pont de Claix, France, agar bacteriological from Oxoid Ltd. (Hampshire, England, UK) potassium phosphate monobasic anhydrous (99.5%) and sodium phosphate dibasic anhydrous (99.5%) were from Merck Germany, and sodium chloride was received from VWR international BVBA, Belgium. All chemicals were used as received. 0.1 M Phosphate buffered saline (PBS) was prepared from 80 g NaOH, 2 g KCl, 14.4 g anhydrous  $\text{Na}_2\text{HPO}_4$ , 2.4 g anhydrous  $\text{KH}_2\text{PO}_4$  in 1000 mL R.O. water, which was adjusted to pH 7.4 if needed, and autoclaved at 121 °C for 15 min. Milli-Q water was used throughout the experiments, with the exception of the bacterial attachment tests, for which sterilized reverse osmosis (R.O.) water was used.

### 2.2. Membrane Modification and Surface Modification

#### 2.2.1. Flat Sheet Membranes

The flat sheet polyethersulfone membranes (0.2  $\mu\text{m}$  pore size) were incubated for 8 h at pH 5 and  $25 \pm 1$  °C, in 40 mL sodium acetate buffer, containing 28.8 mM 4-hydroxybenzoic acid and 0.5 U/mL enzyme. Air was supplied as  $\text{O}_2$  source and was used for gentle continuous mixing to ensure a homogeneous reaction medium. The liquid was stirred for 1 min before the enzyme was added. After the specified modification time, the membranes were removed from the reaction medium and washed by a strong spray flushing with water, followed by three times dipping in deionized water. The modified membranes were dried in glass dishes placed in desiccators supplied with self-indicating blue silica gel for 24 h before evaluation.

A dead-end stirred filtration cell (Millipore, Model 8050, active transport area 13.4  $\text{cm}^2$ ) was used to characterize the filtration performance of unmodified and modified membranes. Pure water flux was measured at a constant transmembrane pressure of 1 bar at  $24 \pm 1$  °C and 300 rpm. The pure water flux is calculated with Equation (1), in which,  $J_w$  = water flux ( $\text{m}^3 \text{ m}^{-2} \text{ s}^{-1}$ ),  $Q$  = quantity of permeate collected ( $\text{m}^3$ ),  $\Delta t$  = sampling time (s), and  $A$  = the membrane area ( $\text{m}^2$ ).

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

#### 2.2.2. Hollow Fiber Membranes

Hollow fiber polyethersulfone lab modules with one single fiber were kindly provided by Pentair X-flow (0.79 mm inner diameter and pore size that is expected to be around 10 nanometers). For modification, first Milli-Q water was pressurized through the membrane for 20 min (dead end), and the

flux was measured three times at 4 bar. Then, 10 mL of 28.8 mM 4-hydroxybenzoic acid (pH 5,  $24 \pm 1$  °C) was rinsed through the fiber by gravity, 4 cm height difference was applied between both ends of the module; outlet flow  $\sim 1$  mL min<sup>-1</sup>). Next, 20 mL of fresh well-mixed 4-hydroxybenzoic acid and laccase enzyme liquid (28.8 mM and 0.5 U mL<sup>-1</sup>, respectively) were rinsed through the module also by gravity. This procedure was repeated every 15 min, and freshly mixed reactants were rinsed through the module at a total modification time of 2 h. The modified membrane was cleaned by forward and backwashing with Milli-Q water at 4 bar and the flux was measured (see Equation (1)). Next, 1 g L<sup>-1</sup> BSA was pressurized at 4 bar (pH 7, 0.1 M sodium acetate buffer,  $24 \pm 1$  °C) through the membrane in dead-end mode and both flux (after washing with Milli-Q) and concentration of BSA in the outlet were determined. The same procedure was applied for unmodified membranes.

### 2.2.3. Modified PES Surfaces on Silicon Wafers

Silicon wafers (with approximately 70 nm silicon dioxide layer) were cut into strips of  $1 \times 5$  cm (static conditions) or  $1.5 \times 5.5$  cm (dynamic conditions). The strips were sonicated in ethanol for 15 min, washed with water and ethanol, and dried in flowing nitrogen. Subsequently, the strips were given a plasma treatment (PDC-32G, Harrick at RF-level high) for 10 min. Immediately after plasma cleaning and removal of any dust by using a flow of nitrogen, the strips were spin coated with 0.25% wt. PES solution in dichloromethane for 10 s at 2500 rpm. The PES coated strips were put at 300 °C for 60 min. The spin-coated PES surfaces were then incubated in 20 mL ( $1 \times 5$  cm strips) or 33 mL ( $1.5 \times 5.5$  cm strips) of 0.1 M sodium acetate buffer containing 28.8 mM 4-hydroxybenzoic acid and laccase (0.5 U mL<sup>-1</sup>), at  $23 \pm 2$  °C and pH = 5. A flow of air was used for mixing and as oxygen source. After completing the reaction, the modified surfaces were removed from the liquid and washed by strong flushing with Milli-Q water. The modified PES surfaces were kept 24 h in glass-covered dishes in desiccators supplied with silica gel for drying.

## 2.3. Bacterial Adhesion and Biofilm Formation

### 2.3.1. *Listeria Monocytogenes* Strains and Culture Conditions

*L. monocytogenes* strains EGD-e and LR-991 were stored in brain heart infusion (BHI) broth (Becton Dickinson, Le Pont de Claix, France), containing 15% (v/v) sterile glycerol (Fluka, Buchs, Switzerland) at  $-80$  °C. Single colonies were inoculated in 10 mL BHI in 50 mL tubes (Greiner Bio-One, Frickenhausen, Germany) and were grown overnight (static incubation) at 30 °C. Overnight-grown cultures were used to inoculate (1%) 25 mL BHI in 50 mL tubes and incubated for 24 h at 30 °C. Cells were harvested by centrifugation at 5000 rpm for 15 min at 20 °C and subsequently resuspended in 1 mL PBS (attachment) or BHI (biofilm formation).

### 2.3.2. Attachment and Biofilm Formation

#### 2.3.2.1. Static Conditions

To determine attachment of bacteria, modified PES slides were immersed in 20 mL PBS in petri dishes and the bacterial suspension was added (approximately 10<sup>9</sup> total cfu). The PES slides were

incubated for 2.5 h at room temperature ( $23 \pm 2$  °C). After washing twice in PBS, the adhered bacteria were collected using a sterile cotton swab. The swab was then placed in 1 mL PBS and vigorously vortexed. The suspended bacteria were serially diluted in PBS and plated. The plates were incubated at 30 °C for 48 h and colonies were counted. Experiments were performed in duplicate in two biological independent replicates. Biofilm formation on the modified PES slides was determined following a similar procedure, with the exception that PES slides immersed in BHI were incubated for 24 h.

#### 2.3.2.2. Dynamic Conditions

Bacterial attachment under dynamic conditions was tested using a flow cell. A  $1.5 \times 5.5$  cm size slide was loaded on the sample support inside the flow cell (sample support size: 1.6 cm width  $\times$  5.7 cm length  $\times$  1 mm depth). The bacterial suspension was diluted in 500 mL PBS. Prior to each experiment (approximately  $10^9$  total cfu), the whole system (connection tubes and the flow cell) was washed with PBS for 10 min and subsequently filled with the bacterial suspension. The bacterial suspension was circulated ( $0.038 \text{ m s}^{-1}$ —Reynolds number is 38 in case of water at 20 °C) through the system for 2.5 h. The PES slide was recovered and bacteria were harvested and counted as described for static conditions. Experiments were performed in three independent biological replicates.

#### 2.3.2.3. Statistics

Microsoft Excel was used to apply t-test on the obtained data; differences were considered to be statistically significant at  $P < 0.05$ , which was the case for all samples compared to the blank surface measured at the same conditions.

### 2.4. Membrane and Surface Analysis

#### 2.4.1. Grafting Yield

The amount of material grafted onto the membrane surface was calculated from the weight of the membrane, before and after grafting; 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 self-indicating blue silica gel to remove any moisture. To remove any loosely bound material, the weight of the membrane after grafting was measured after washing the membrane by filtration of at least 1000 mL deionized water, and subsequent drying in the desiccator.

#### 2.4.2. BSA Adsorption

BSA was used as a model compound to evaluate protein adsorption on unmodified and modified membranes.  $1 \text{ g L}^{-1}$  BSA solution at pH 7 was prepared using 0.1 M sodium acetate buffer. The membranes were immersed in 50 mL BSA solution and gently shaken (200 rpm) at 25 °C for 24 h. BSA concentration in the solution was measured using a UV-Vis spectrometer (280 nm), and from this, the adsorbed amount was calculated. After completion of the experiment, each membrane was intensively washed, first by spray flushing three times, then followed by repeated (3 $\times$ ) immersion in deionized water and decantation.

The membrane flux after BSA adsorption was determined by backwashing the membrane, using 100 mL of deionized water to remove any unbound BSA, and then forward washing, using 100 mL of

fresh deionized water. After that, fresh deionized water was used in forward motion at 1 bar applied pressure, and the flux was determined in three independent measurements, the data presented in Table 1 are the averages of these measurements.

#### 2.4.3. Fluorescence Microscopy for Bacterial Adhesion

Fluorescence microscopy experiments were performed on a BX41 microscope (Olympus, Zoeterwoude, The Netherlands). Images were acquired using a XC30 camera (Olympus) and Olympus Cell<sup>^</sup>B software. After washing the modified PES slide twice with PBS, it was placed on a microscope slide (76 × 26 mm), and a square cover glass (18 mm) was placed on top of the sample. Green fluorescent protein (GFP) was visualized using a MNIBA3 filter (Olympus).

### 3. Results and Discussion

#### 3.1. Performance of Flat Sheet Membranes

In previous publications [6], we have reported on the performance of enzyme-modified flat sheet membranes, and found that the average flux reduction was mostly below 10% when 4-hydroxybenzoic acid was used as the modification agent (specifications on the modification procedures are given in the experimental section; performance of hollow fibers is described in the next section). Besides, it was found that, longer modification times (*i.e.*, hours) and higher substrate concentrations lead to modified membranes with better protein repellence, and we contribute that to brush formation on the surface (see Figure A1 in Appendix), of which the effectiveness is linked to the graft density and length of the polymer brushes [9]. Under some reaction conditions (e.g., 28.8 mM 4-hydroxybenzoic acid, pH 5, 20 °C, enzyme concentration 0.5 U mL<sup>-1</sup>), protein adsorption was even prevented. Further, the mechanical and thermal properties of the membrane were not adversely affected by the modification method.

We concluded that laccase-catalyzed modification of PES membranes is a mild method with low environmental impact given that the only reaction product that is formed is water. The method leads to effective protein repellence while keeping the bulk properties of the base membrane intact. This all makes laccase-catalyzed modification an interesting alternative for currently used membrane modification methods, and in this paper we take these two steps further by modification of hollow fiber membranes and investigating bacterial adhesion on modified surfaces. We chose to work under the same reaction conditions that were used to investigate protein repellence in hollow fiber modules. Further, we tested bacterial adhesion on similarly modified surfaces, since protein adsorption is often considered the initial step for bacterial adhesion. Finally, we conclude with an economic analysis of the modification method.

#### 3.2. Performance of Hollow Fiber Membranes

The hollow fiber modules were purposely built by Pentair X-flow and consisted of one fiber of which the pore size was estimated to be around 10 nm. These membranes had considerably smaller pores than the microfiltration membranes we used in earlier work and had pores of 0.2 μm.

Table 1 shows an overview of the results obtained with two sets of membranes, one modified and the other not. The results are in good agreement, and the procedures are reproducible as was also the case in previous work with flat sheet membranes. However, there are also notable differences with previous work, namely the flux reduction through modification is much greater (70%–80%, compared to less than 10% for microfiltration membranes). Obviously, this is related to the pores size. The modification layer will be relatively large compared to the pore size in the hollow fiber membranes, especially when compared to the microfiltration membranes that were used in previous work. Upon exposure to BSA, the flux of the unmodified membrane reduced to approximately 12% of its original value. Slightly higher fluxes were found compared to the unmodified membrane, while the relative flux decrease due to BSA flushing through the modified membrane was much less. The measured fluxes during BSA flushing were similar for modified and unmodified membranes.

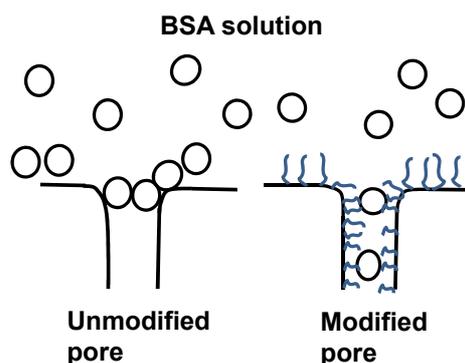
**Table 1.** Specification and performance of the unmodified and modified high flush surface hollow single fiber polyethersulfone (PES) lab modules.

Parameter	Unmodified	Unmodified	Modified	Modified
Length (cm)	23.7	24	23.6	24
Area (cm <sup>2</sup> ) *	5.88	5.96	5.86	5.96
Flux (m <sup>3</sup> m <sup>-2</sup> h <sup>-1</sup> )	0.3 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
After modification (m <sup>3</sup> m <sup>-2</sup> h <sup>-1</sup> )			0.072 ± 0.001	0.1 ± 0.001
BSA flux (m <sup>3</sup> m <sup>-2</sup> h <sup>-1</sup> )	0.04 ± 0.001	0.039 ± 0.001	0.043 ± 0.001	0.046 ± 0.001
BSA rejection (%)	99 ± 0.8	99.1 ± 0.8	50.7 ± 0.9	50 ± 2.5
Residual flux after BSA (m <sup>3</sup> m <sup>-2</sup> h <sup>-1</sup> )	0.04 ± 0.001	0.04 ± 0.001	0.043 ± 0.001	0.043 ± 0.001

\* Membrane area depends on the length of the potting material, which varies slightly.

The most striking difference between the membranes is in their rejection of BSA which was around 50% for modified membranes and almost 100% for the unmodified membrane. The unmodified membranes accumulated protein on their surfaces, which reduced the pore size and increased rejection. The lower rejection of the modified membranes is expected to be a result of reduced protein adsorption on/into the membrane pores, as is schematically illustrated in Figure 1, which allows passage of the protein through the membrane.

**Figure 1.** Schematic representation of the effect of modification of polyethersulfone (PES) membrane pores on rejection of proteins.



This is an interesting aspect, since it allows us to start from a chemically stable base membrane, and modify its properties systematically based on the amount of material used for modification. Please note that protein repellence from the membrane surfaces is different from protein rejection by the membrane; a perfectly protein repellent surface will only correlate with a completely nonrejecting membrane if the pore size allows unhindered passage of the protein.

### 3.3. Bacterial Adhesion

We also tested those modified surfaces that showed good protein repellence [6] for their effect on bacterial adhesion, because adsorption of cellular surface proteins is often considered to act as an initial step in microbial adhesion. We used *Listeria monocytogenes* as a model microorganism, and tested its ability to adhere and grow on modified PES surfaces under static and dynamic conditions. *L. monocytogenes* strain EGD-e is the most commonly used research strain worldwide, and LR-991, showed the highest biofilm formation in a screen of 143 *L. monocytogenes* strains [10].

Under static conditions, the ability of modified PES surfaces to resist the attachment of *L. monocytogenes* cells from EGD-e and LR-991 strains grown at 30 °C was determined (Table 2;  $P < 0.05$  for all samples compared to unmodified membrane). The results show approximately 60% reduction in the number of attached Listeria cells from both strains relative to the numbers that can adhere and grow on the unmodified surface; this can most probably be attributed to the brush-like structure of the modification layer. Possibly the effect is similar to what has been demonstrated in other studies [11–13], where the brush-like structure of the modifying layer on different surfaces, such as stainless steel, glass, polyamide and polyester, also reduced bacterial adhesion. The effectiveness of these structures against bacterial attachment is caused by steric hindrance that keeps the bacterial cells at a distance from the surface, which results in weakening of the (e.g., van der Waals) interactions [13].

**Table 2.** Reduction in bacterial adhesion on modified surfaces.

Tested strain and conditions	Attachment	Growth
<b>EDG-e strain</b>		
Static conditions	57% ± 5.3	63% ± 2.4
Dynamic conditions	93% ± 0.2	–
<b>LR-991 strain</b>		
Static conditions	59% ± 6.0	63% ± 2.0
Dynamic conditions	88% ± 1.7	–

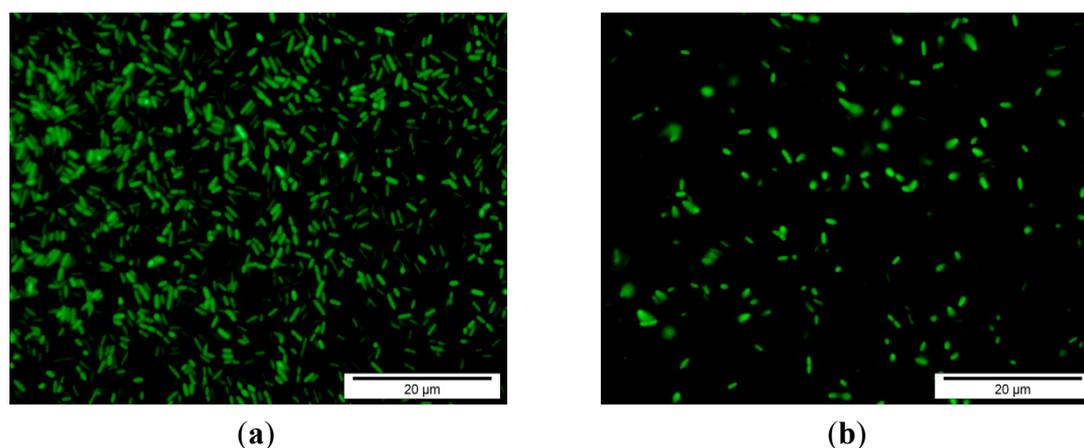
Note: Standard deviations were obtained for four model surfaces, using two separate bacterial cultures.

An impression of the difference in attached cells was also obtained with fluorescent microscopy, as illustrated in Figure 2. Although these images are to be treated with care, since they can give a false impression of the actual effect that is reached due to the relatively small sampling area, they qualitatively corroborate the reduction in the Listeria adhesion on the modified model surfaces relative to the blank.

Compared to static conditions, dynamic conditions show an even greater reduction in adhered bacteria from both strains. It seems that if bacteria are not immediately attached to the surface, they can be carried away from the surface by the cross-flowing liquid, possibly as a consequence of lift

forces that may even prevent them from coming into frequent contact with the surface [14]. However, at the blank surface, considerable amounts of bacterial cells are present, indicating that the modification layer has a significant role in the reduction. This could be a direct effect on prevention of adhesion, or in a delay of adhesion (less fast) that allows lift forces to remove loosely adhered bacteria.

**Figure 2.** Examples of fluorescence images of the EDG-e strain of *Listeria monocytogenes* attached to unmodified (a) and modified (b) PES surfaces using static conditions.



### 3.4. Cost Analysis

Although prevention of adhesion and adsorption in hollow fiber membranes is not absolute, we like to interpret the effects that we found in a positive way; there is still plenty of room for improvement! Obviously this also still needs to be part of our ongoing research, but before going any further, we first need to consider the costs related to enzymatic modification of membranes, and check if this is a realistic option.

Both the enzyme and the used substrates are available commercially and are not expensive, and it is possible to recycle the enzyme, substrate, and buffer. Table 3 shows an indication for the price of modification of one square meter of membrane with 4-hydroxybenzoic acid (28.8 mM, 0.5 U mL<sup>-1</sup> enzyme) according to prices in the Sigma/Fluka catalogue 2011. Based on the prices of the largest packages available in the catalog, the cost of modification chemicals is 120–130 euro per m<sup>2</sup>.

**Table 3.** Price indication for modification of 1 m<sup>2</sup> of polyethersulfone membrane.

Material	Sigma/Fluka 2011	Used amount/0.002 m <sup>2</sup> membrane	Membrane cost per m <sup>2</sup> (€/m <sup>2</sup> )
Sodium acetate	43.1 €/1 kg	0.11 g	2.4
Acetic acid	13.5 €/1 L	0.04 mL	0.3
Enzyme	38.6 €/1 g	6 mg	115.4
4-hydroxybenzoic acid	48 €/1 kg	0.2 g	4.8
Total			122
On industrial scale the price will be 10 times lower, leading to a price per m <sup>2</sup>			12.2

Note: The costs related to oxygen needed for modification are negligible.

From the list it is clear that the enzyme costs are prominent, and when produced at large scale, also this cost will be reduced considerably. For a commercial enzyme that is, for example, used in washing

powder, a typical price would be 100 €/kg of crude enzyme. The activity of such enzyme will be less than the one we used in our experiments, but even if the enzyme activity has a factor of 10 lower, the price for modification would be reduced by a factor of approximately 50; alternatively, the reaction could also be carried out at lower enzyme concentration, although this would require longer reaction times. In summary, it is realistic to say that for larger scale production the price can be reduced by at least a factor of 10 compared to the Sigma/Fluka catalogs, leaving a calculated cost of approximately 12 €/m<sup>2</sup>.

Besides the direct costs illustrated in Table 3, enzyme catalyzed modification has many positive aspects. To name a few, the method is simple, and does not require specific expensive equipment. It can be carried out under very mild conditions (with low impact on the environment), and also on hollow fiber modules, which is an important step for large-scale application. The method is reproducible as illustrated in the grafting yield (e.g., [4–6]) and performance shown. Apart from this, the residual flux after modification is high, and especially protein repellence is an important feature of these modified membranes. Together with the stability of the modification layer at low pH and to some extent at high pH, this may also lead to less replacement costs.

#### 4. Conclusions

In this paper we have shown that the necessary steps to make enzymatic modification of polyethersulfone membranes feasible for large-scale application are within reach. The modification conditions that we found effective in rejecting proteins from microfiltration membranes also proved to influence protein adsorption in hollow fiber membranes. Besides, the pore size of ultrafiltration membranes could be adjusted partially with the proposed modification method.

Also in regards to bacterial adhesion, the previously identified modification conditions showed interesting results leading to 90% reduction of *Listeria monocytogenes* attachment under dynamic flow conditions. Moreover, the costs related to enzymatic modification are realistic, and all these aspects make us believe that there is a bright future for this modification method.

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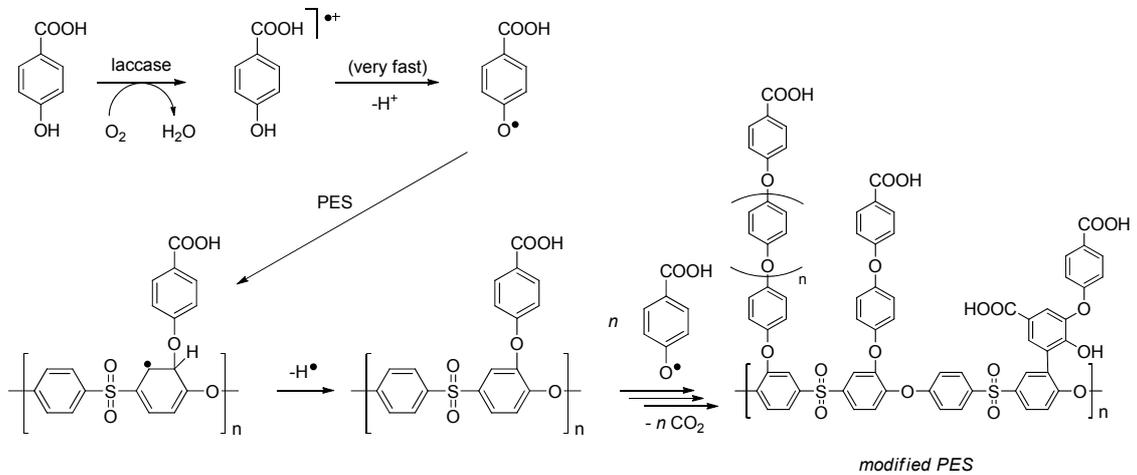
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## Appendix

**Figure A1.** Tentative mechanism for the formation of reactive 4-hydroxybenzoic acid radicals by laccase and grafting of the radicals to PES membranes.



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