Enzyme-Catalyzed Modification of Poly(ethersulfone) Membranes

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This research was conducted under the auspices of the Graduate School of VLAG

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Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 21 March 2012
at 1.30 p.m. in the Aula.

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Enzyme-Catalyzed Modification of Poly(ethersulfone) Membranes.
172 pages.

Thesis, Wageningen University, Wageningen, NL (2012)
With references, with summaries in English, Dutch, and Arabic.

ISBN: 978-94-6173-145-6

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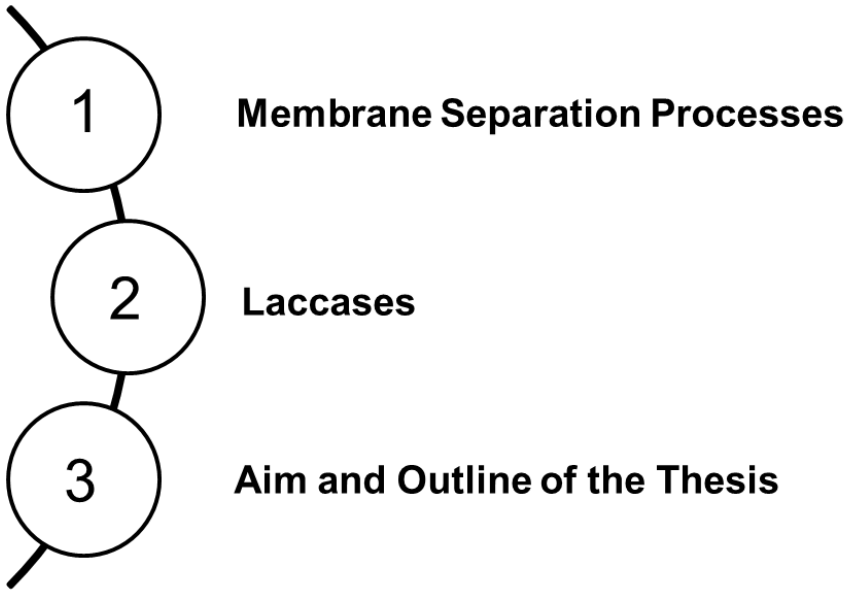
Chapter One

General Introduction



General Introduction

In this chapter, two essential parts of this thesis will be introduced. First, a short description of membrane processes will be given, including some drawbacks that can be hurdles in their application, such as protein adsorption and microorganism adhesion. To influence these effects, membrane modification was investigated. In contrast to (grafting) methods known from literature, the method presented in this thesis uses the enzyme laccase, which allows better control over the modification process and enables an essentially mild method. Therefore, the second section of this introductory chapter is devoted to laccases. The chapter is concluded with the description of the rationale behind the research, and an overview of the content of the various chapters.

1. Membrane Separation Processes

Membrane-based processes have become an important unit operation for a wide range of industries and are used for separation, fractionation, concentration, and/or purification of (molecular) mixtures. Some illustrative examples can be found in the manufacturing of dairy products, water treatment to remove bacteria or salt (desalination), and dialysis to clear the blood of people suffering from kidney disease. In addition, novel applications have been published, *e.g.* membrane contactors and membrane (bio) reactors and micro reactors. The main advantages of membrane-based technology are the possibility of ambient temperature operation (therewith reducing damage to temperature sensitive components), relatively low capital and running costs, and modular construction (see Figure 1) [1].

Figure 1. Membrane module array used in water treatment plant in Singapore.

Although membranes are promising, there are a number of practical limitations for their use, such as concentration polarization and membrane fouling. In this thesis, membrane fouling is defined as the irreversible accumulation of substances on the membrane surface and/or within the membrane pores, which results in deterioration of membrane permeability (flux) during operation [2,3]. Away from this, also (partly) reversible effects such as cake formation will influence the flux of the membranes negatively, but because this effect can be controlled through appropriate process conditions, we will not discuss this in detail. Also membrane compaction may occur, but this mostly occurs in high pressure processes such as reverse osmosis and gas separation, and this is considered outside the scope of the thesis.

In the following section, concentration polarization and fouling (more specifically by proteins and microorganisms, which are both highlighted in this thesis) are introduced, including membrane materials that are frequently applied.

1.1. Concentration Polarization

Rejection of certain components by the membranes leads to accumulation of components that cannot pass the pores, resulting in an increase in concentration close to the membrane. This then gives a driving force for transport from the component in the (laminar) layer next to the membrane surface to the bulk liquid. The balance between the component carried toward the membrane by the applied transmembrane pressure, and back transport due to the concentration difference is called concentration polarization. Concentration polarization can be controlled by decreasing the operating pressure, or increasing the cross-flow velocity of the feed solution. If components present in the concentration polarization layer attach to the membrane, this can be seen as the initial step of fouling [4,5]. Various components are known to show high affinity to surfaces, such as proteins, polysaccharides, but also small components - such as minerals (that cause scaling) and 'large' microorganisms (that are able to form biofilms) - are known to have a detrimental effect on membrane performance.

1.2. Membrane Fouling

As defined earlier, membrane fouling is the irreversible deposition of components on and into the membrane (see difference between fouled and un-fouled membrane in Figure 2). In literature, it is stated that fouling depends on several factors, such as the membrane material which determines the physicochemical interactions between the membrane and the substances in the feed solution [6,7] (see membrane material section). Obviously, the properties of the substance are of importance, because they determine to a large degree whether a component can attach to the membrane surface.

Various components have been described to cause fouling, such as colloidal particles [8,9], minerals that cause scaling [10,11], antifoam [12,13], proteins [14,15], and microorganisms [16,17]. Also the location of fouling can be very different, ranging from surface deposition to in-depth fouling. As a result, various 'solutions' have been proposed to influence the interaction between these foulants and membranes, ranging from adjustment of the system hydrodynamics [18-20], to surface modification [21,22], and downright regular cleaning [23,24]. However, none of these methods is able to truly prevent fouling. Especially in-depth fouling is very hard to remove because the foulant will also partially block the local flow that is needed to remove and carry away the foulant. Perhaps even more relevantly, once a foulant is attached to the membrane, it works as an initiator for attachment of more foulants. For example, protein adsorption can be an initial step for attachment and growth of microorganisms (*i.e.*, biofilm formation).

Figure 2. Fouled (right) and un-fouled (left) spiral wound membranes that are opened up for inspection; the left membrane is light in color inside the unwrapped module (not to be confused with the module holder which is yellow/grey in color), where the right membrane is dark brown all over.

In this thesis, the main focus will be on influencing/preventing protein adsorption, and some of the modified surfaces that showed promising results were used to investigate interaction with polysaccharides, polyphenols, and microorganisms (see next sections).

1.2.1. Protein Fouling

Proteins have both hydrophilic and hydrophobic regions, the ratio being different for different proteins. It is often postulated that increased membrane hydrophilicity is the main tool to mitigate protein fouling, the main reason being that the hydrophilic surface prefers water in its neighborhood and this reduces adsorption of proteins [25]. However, not only the surface hydrophilicity plays a role in protein repellence, but also charge on the protein and structural changes in the protein [26,27]. Besides, the membrane surface charge and structure have a significant impact on prevention of protein adsorption and attachment of other foulants [25,28,29]. In order to influence membrane structure, polymers and monomers have been grafted to membranes [30] or to the polymer from which the membrane is prepared [31]. Alternatively adsorption of block co-polymers has been proposed [32]. Grafting is mostly initiated with a glow discharge apparatus or by UV irradiation [33,34]. In some cases this leads to additional charge on the surface (monomer grafting) or to addition of polymer chains onto the surface, and these chains may act as a steric hindrance for proteins that are close to the membrane surface. In this thesis we succeed in attaching (variously sized) polymer chains to the membranes by enzymatic grafting, and created an effective barrier for foulants.

1.2.2. Biofouling

In general, biofouling is defined as the attachment and/or growth of cells *e.g.* microorganisms and algae on surfaces. Biofouling occurs on all kinds of surfaces once adequate circumstances such as the presence of nutrients for cell adhesion and growth are available. Biofouling is known to cause serious problems in/on medical devices, on ship hulls, in/on pipelines, receivers, etc. [35], and it is also an important factor in membrane processes [35-37].

In membrane biofouling three main steps can be distinguished [16,38]: (1) adsorption of macromolecules *e.g.* proteins, (2) primary adhesion by fast-adhering cells, and (3) colonization and growth with development of a biofilm, which ultimately leads to irreversible blocking of the membrane. In a next step, cells may be expelled locally, and colonize on a different part of the membrane. Biofouling is a complex process that is reported to be affected by many factors, including the characteristics of the micro-organisms, membrane surface properties (*i.e.*, membrane material, charge, roughness, shape, etc.) and environmental factors such as pH, ionic strength, etc. [17,39].

Generally, two strategies are used to control biofouling (to some extent) in membrane processes [40]; (i) optimization of operating conditions, including pretreatment of feed and cleaning procedures, and (ii) membrane modification [37,41,42], *e.g.* through grafting, coating, etc. [37,40,43]. In general, it is believed that more hydrophilicity, negative charge, and smooth surfaces reduce the initial adhesion of microorganisms [35,40,44].

1.3. Membrane Materials

Membranes can be made from various materials, ranging from inorganic materials to (bio-) polymers; examples are shown in Figure 3. From the material of choice, it should be possible to form the membrane structure in a controlled way, and this limits the options for membrane production considerably. Chemically and thermally stable membranes are understandably targeted by membrane manufacturers, even if these membranes are not intrinsically the most resistant ones against fouling. Mostly this is mediated by an after-treatment that functionalizes the membrane further, as is also done in this thesis, starting from poly(ethersulfone).

Popular polymeric membrane materials may take biopolymers as a starting point such as cellulose acetate (CA), cellulose nitrate, regenerated cellulose, or synthetic polymers *e.g.*, polypropylene (PP), polyethylene (PE), polyamide (PA), polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF), polysulfone (PSf), poly(ethersulfone) (PES), polyimides (PI), polyetherimids (PEI), polyacrylonitrile (PAN), etc. Inorganic membranes can be made of

ceramics, metal, glass or carbon, and recently even silicon wafers have been used in so-called microsieves. Also hybrid membranes, which consist of polymeric and inorganic materials together, have been presented [21,22,28,45-47].

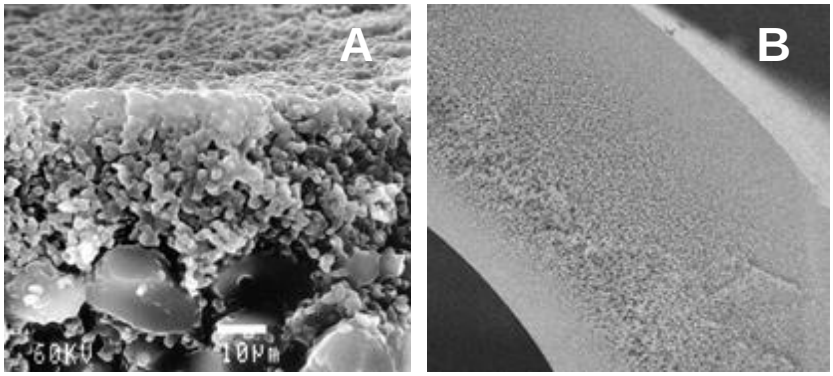


Figure 3. Membrane materials: (A) Inorganic ceramic and (B) polymeric.

Membranes can have many different properties. Here we briefly discuss the development of cellulose acetate membranes. Early cellulose acetate (38-40% acetyl content) membranes were dense (symmetric) films. Later, cellulose acetate membranes were prepared with asymmetric structures by Loeb and Sourirajan, and these were used in the first industrial application on desalination [2,48]. The incorporated swelling agent, *e.g.* $Mg(ClO_4)_2$ and formamide in an acetate-acetone solution, gelled the membrane in a low-temperature bath. Subsequently, the membrane was treated in water at 60-90 ºC, which forms a rejection layer on a more open carrier membrane [48]. The effects of preparation procedure, chemical composition, and annealing temperature on the properties and performance of cellulosic membranes have been widely studied [48,49], and it is clear that the preparation conditions are of great influence on the resulting membrane. Currently, cellulose acetate membranes are readily used in reversed osmosis, nano- and diafiltration. The main advantages of cellulosic membranes are their low price and the fact that they are hydrophilic, which makes them less prone to fouling. On the other hand, the mechanical and thermal stability of the polymer are not that great, and the membrane is susceptible to (bio) fouling, which makes use of other artificial materials attractive [3,50].

Nowadays, synthetic polymers are often used in membrane preparation, because of their resistance to chemical agents and/or heat [2,3,21]. For example, polyamide can withstand higher temperature and larger pH variations (4-11) than cellulose acetate, but is more prone to damage by chlorine and oxidation, and is more expensive. Among the most popular membrane materials are poly(arylsulfone)s, such as PSf and PES, which can be processed

well and allow preparation of membranes in different shapes. The relatively high glass transition temperature of the material allows steam sterilization, which - in combination with good chemical stability - makes it an interesting material for membrane preparation, in spite of the relatively high price. The hydrophobic character of the material is partly mediated by blending with polyvinylpyrrolidone (PVP), which also allows for a wider variation in pore size [21,22,28]. Unfortunately, poly(arylsulfone) membranes still show a high binding affinity for different molecules such as proteins and microorganisms, which causes severe fouling of membranes during operation. To diminish such fouling, various methods have been proposed to alter the surface properties of poly(arylsulfone) membranes, and to reduce adsorption of different foulants [21,22]. An overview of the various methods used for modification of poly(arylsulfone) membranes is given in chapter two of this thesis. In general, the various suggested methods are effective to some extent, but are typically environmentally adverse. Clearly, there is still a lot of room for improvement, also regarding the reproducibility of the modification methods.

In this thesis, poly(ethersulfone) (PES) membranes will be modified using the enzyme laccase as initiator for free radical formation, which leads to covalently attached (poly)phenolic components of various sizes and shapes, as described in the various chapters. The enzymes that catalyze the modification of such so-called ‘chemically inert’ membranes under mild conditions are introduced in the next section.

2. Laccases

Enzymes differ from ordinary chemical catalysts in several aspects such as mild reaction conditions, and high reaction specificity [51]. Six classes of enzymes are distinguished: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Oxidoreductases are enzymes that catalyze the transfer of one or more electrons from one molecule (the reductant, or electron donor) to another molecule (the oxidant, or electron acceptor). In case of oxidases, they catalyze oxidation reactions involving oxygen as electron acceptor, thereby reducing oxygen to water or hydrogen peroxide. Catechol oxidases, tyrosinases, and blue copper oxidases - including laccases, ascorbate oxidases, and ceruloplasmin - are examples of different types of oxidases [52]. Several reviews on laccases have been published in recent years, providing excellent summaries of the enzyme kinetics and applications [51,53,54]. Here we summarize the most important points, emphasizing current and future applications of laccases.

Laccases [EC 1.10.3.2] are glycoproteins with a molecular mass ranging from about 50 to 100 kDa [54]. They can be roughly divided into two major groups which show clear differences (see further in this Chapter), *i.e.* laccases from higher plants and those from fungi [53,55]. The first laccase was described by Yoshida at the end of the 19th century as a component of the resin ducts of the Chinese or Japanese lacquer tree *Rhus vernicifera* [56], (recently renamed to *Toxicodendron vernicifluum*), and this makes it one of the oldest enzymes ever described. The name laccase refers to this lacquer tree. Further, laccases have been identified in trees, various vegetables and fruits [53-56], fungi [51,54,57], and bacteria [58-63]. Besides, proteins with features typical of laccase have been identified in insects [64] and prokaryotes [54]. Although known for a long time, laccases only attracted significant attention after studies on enzymatic degradation of wood by white-rot fungi [53,55,58].

2.1. The Catalytic Center and Reaction Mechanism of Laccase

Laccases catalyze the oxidation of a broad range of substrates such as phenolic compounds, and aromatic or aliphatic diamines to the corresponding radical cation, which rapidly lose a proton to give a radical. The redox process takes place with the assistance of a cluster of four copper atoms that form the catalytic core of the enzyme (see Figure 4). Blue copper oxidases contain at least one type-1 (T1) copper ion, which is the primary electron acceptor. Blue multi-copper oxidases typically employ at least three additional copper ions; one type-2 (T2) and two type-3 (T3) copper ions arranged in a tri-nuclear cluster. The latter is the site at which the reduction of molecular oxygen takes place [54,57]. The T1 copper is vulnerable towards solvents [54,65,66].

The redox potential of fungal laccases is in the range of 0.5-0.8 V [67]; plant laccases exhibit redox potentials from 0.3-0.5 V [57,68]. This is a high value for the Cu^{II}/Cu^{I} couple, which normally has a redox potential around 0.15 V in water [65,68]. This is a result of the orientation of the metal ion toward the protein backbone, which forces the metal ion into a strained geometry that leads to a higher redox potential [57,65].

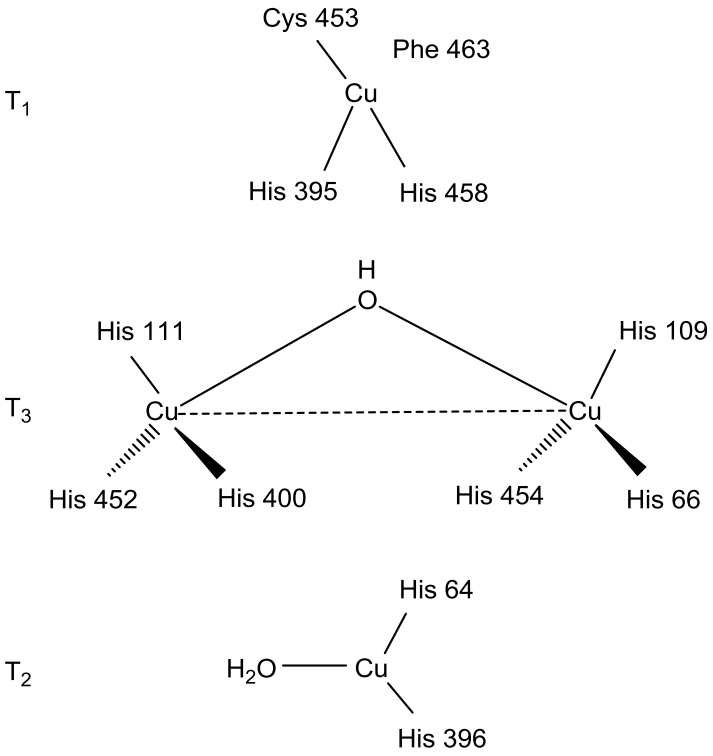


Figure 4. Model of the catalytic center of laccase from *Trametes versicolor* consisting of a cluster of four copper atoms [57].

The first step in the catalytic cycle is oxidation of four reducing substrates by the copper (Cu^{2+} to Cu^{+}) at the T1 site followed by transfer of the electrons from T1 to the T2/T3 tri-nuclear site, resulting in the conversion of the fully oxidized form of the enzyme to a fully reduced state, see Figure 5. The second step is the reduction of dioxygen that takes place via the formation of a bound oxygen intermediate, namely peroxide di-anion that is protonated and splits into the oxy radical and a molecule of water. In the final step, all four copper centers are oxidized again and a second water molecule is released. The intramolecular electron transfer from T1 to the tri-nuclear copper site is rate limiting in the overall reaction, not the electron transfer from the substrate to the T1 copper [69-71].

Oxidations by laccase can be performed directly, *i.e.* the enzyme interacts with the administered substrate itself, or indirectly, in which the enzyme oxidizes a chemical mediator which acts as an intermediate substrate, see Figure 6. The oxidized radical forms of these chemical mediators are able to interact with bulky substrates or compounds having a high oxidation potential [57,72].

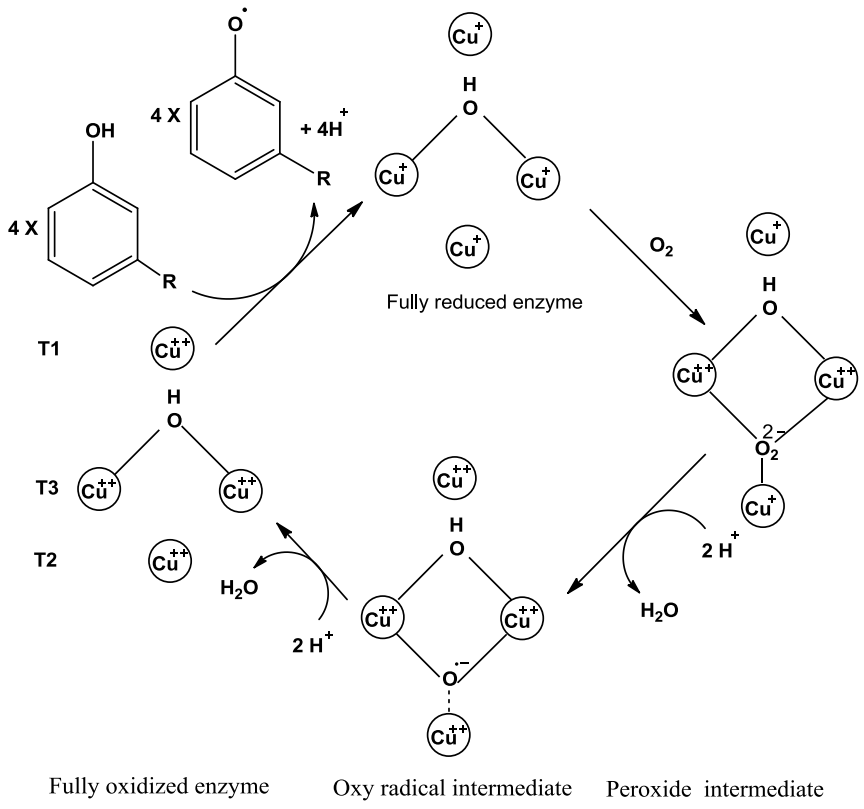


Figure 5. Catalytic cycle of laccase [58,71]

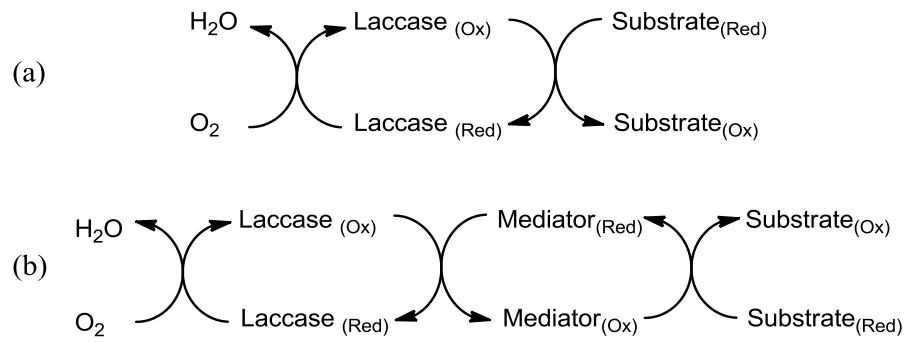


Figure 6. Schematic representation of laccase-catalyzed redox cycles for substrate oxidation in (a) the absence and in (b) the presence of chemical mediators [57].

Although not used in this thesis, for completeness we would like to mention that more than 100 mediator compounds have been described for laccase (see Figure 7 for examples). Here we only mention 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS, which was the first component found. It mediates laccase catalyzed oxidation of nonphenolic compounds, such as veratryl alcohol to the corresponding aldehyde [73,74]. Various laccases readily oxidize ABTS to the cation radical $ABTS^{+\cdot}$ which is intensely green-blue colored (ε_{420} = 36000 $M^{-1}cm^{-1}$) and is often used in activity assays. The redox potentials of $ABTS^{+\cdot}$ and

$ABTS^{2+}$ were evaluated as 0.680 V and 1.09 V, respectively [75]. In general, synthetic mediators are toxic, expensive and mostly inactivate laccase at concentrations above 1 mM; however, also natural mediators such as *p*-coumaric acid, 4-hydroxybenzoic acid, and syringaldehyde [76] have been identified [77] and these components are expected to lead to less negative side-effects.

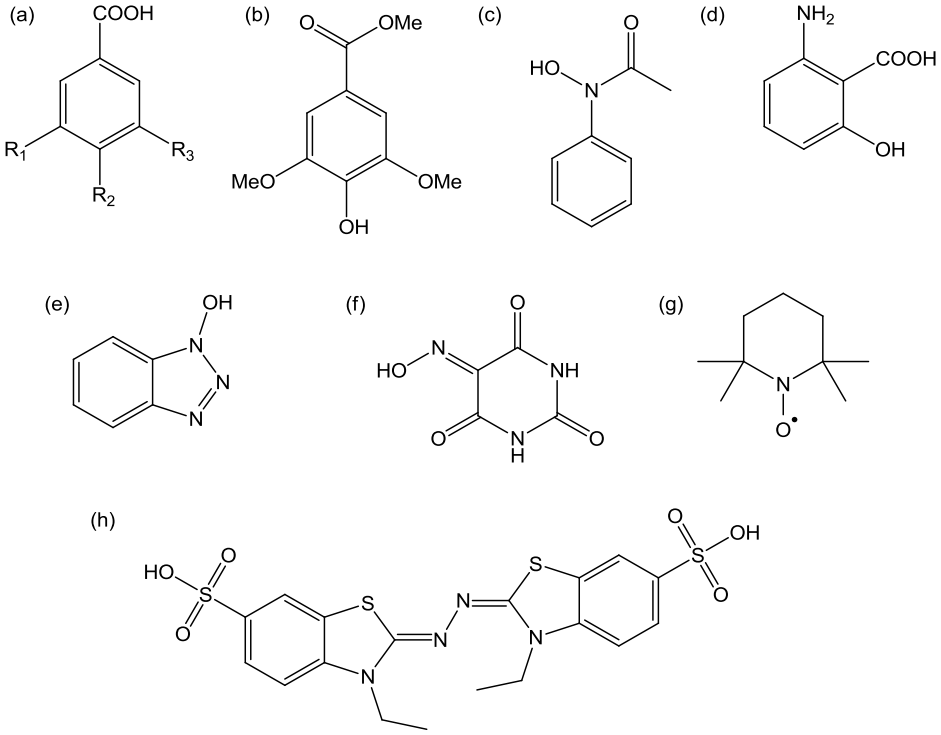


Figure 7. Examples of laccase mediators. (a) benzoic acids, R_1, R_2, and R_3 is OH or COOH or OMe; (b) methyl ester of 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid); (c) N-hydroxyacetanilide; (d) 3-hydroxyanthranilic acid; (e) N-hydroxybenzotriazole; (f) violuric acid; (g) (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO); (h) 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).

2.2. Applications

As mentioned, laccases produce free radicals from suitable substrates using the oxygen from air as an oxidant and producing water as the only by-product. The ensuing secondary reactions are responsible for the versatility of laccases, and this in combination with its thermal stability, its lack of substrate inhibition and high rates of oxidation (10-100 fold higher than lignin peroxidase or manganese peroxidase) make laccase an ideal candidate for the development of enzymatic oxidation processes [58,78].

Laccases have been applied in a variety of processes, such as the clean-up of herbicides, pesticides [79-82], certain explosives and polycyclic aromatic hydrocarbons in soil [82,83].

Furthermore, they have been used in paper processing [84-86] and as cleaning agent for water purification systems [87-89]. Besides, laccases have been applied in organic synthesis for the oxidation of alkenes to aldehydes and ketones [90], and for the dimerization of steroids [91], phenols [92,93], hydroxystilbenes [94], and cyclic alcohols [95]. The most striking examples of laccase applications are summarized in the next section: delignification and pulp bleaching, organic synthesis, bioremediation, and biofuel cells with special attention for surface modification.

2.2.1. Delignification, Organic Synthesis, Bioremediation, and Biofuel Cells

A lot of effort has been put into exploring fungi for technical lignin removal in the pulping process and for bio-bleaching [96]. Two of the most important and best examined lignin degrading microorganisms are the white-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* [51,73]. Laccase is abundant in theses fungi, while it is absent in brown-rot fungi [97]. Compared to conventional ozone delignification, pre-treatment of wood pulp with laccase is milder and cleaner, and less damaging to cellulose [96,98]. Further, it was found that this enzyme can be used for cross-linking and functionalization of lignocellulose compounds for *e.g.* paper-boards [99].

In organic synthesis, laccase has been used in the oxidative dimerization of phenolic derivatives such as tetrahydronaphthol [95], 17β-estradiol [91,95], totarol [93], hydroxystilbenes [92,94], flavonols [100], salicylic esters [101], and recently the flavonolignan silybin [102], which is widely used in human therapy of liver dysfunctions. In addition, laccase was used in the production of aminoquinones at high specificity through amination of p-hydroquinones without formation of hydroquinonoids [103]. Moreover, larger molecules have been produced; polymerization with laccase is considered a green synthesis route with great flexibility [104]. Just to name some examples, laccase shows remarkable activity and stability under acidic conditions in the synthesis of polyaniline [105]. Likewise, laccase was used in the synthesis of a poly(allylamine) catechin conjugate, which is a good antioxidant [106]. The interested reader can see some of these monomers and dimers in appendix A.

In bioremediation processes, laccase is used to protect the environment from damage caused by industrial effluents, through *e.g.* direct oxidation of phenol derivatives. The polymeric polyphenolic derivatives that result from the laccase-catalyzed coupling are usually insoluble and can be separated easily by filtration or sedimentation [107]. Laccase has been used for direct dechlorination of chlorophenolic compounds [108,109] and for detoxification

through conjunction with natural phenols (*e.g.*, syringic acid) [110]. Similarly, poly(catechol) was formed by laccase [89], which can be removed from wastewater streams in the form of a precipitate. This polymer can be used further for selective separation processes and in biosensor applications.

Interestingly, laccase has been successfully used in the presence or absence of a chemical mediator in a dihydrogen/dioxygen biofuel cell to overcome slow reduction of dioxygen to water [111,112].

2.2.2. Surface Modification

The laccase from *Trametes hirsuta* was used to coat flax fibers and fabrics with hydroquinone and various methoxyphenols to obtain antibacterial surfaces; the combination of ferulic acid and hydroquinone resulted in a coating with antibacterial effect against *Bacillus subtilis* and *Staphylococcus aureus* [113]. Also wool was treated with laccase to incorporate water insoluble lauryl gallate to provide antioxidant, antibacterial and water repellent properties to the textile material [114]. Besides, cellulosic fibers can be colored by grafting natural flavonoids, which can be carried out without bleaching, resulting in a more environmentally friendly process [115,116]. Alternatively textile can also be de-colored by the enzyme [77].

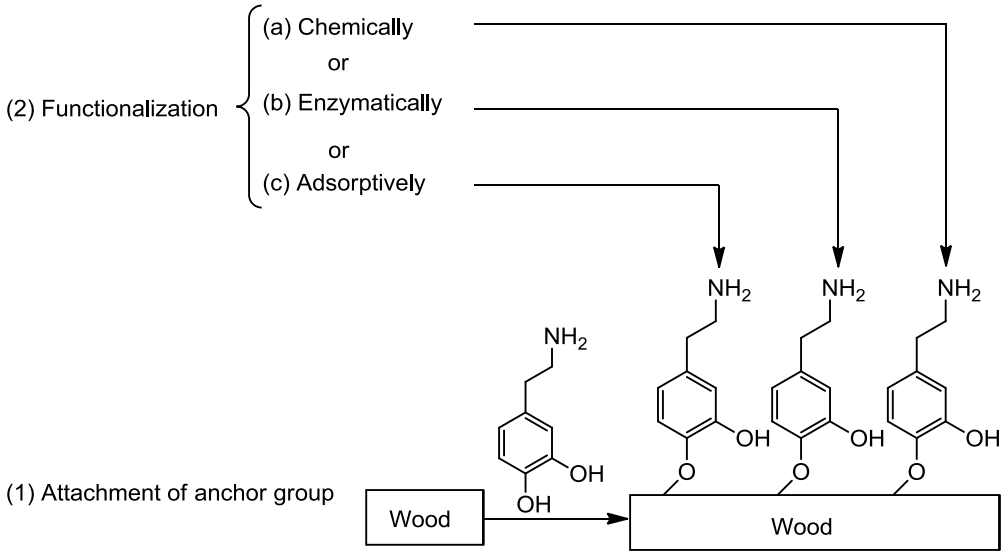


Figure 8. Attachment of phenolic amines to lignin moieties of wood, which can be used for further functionalization [119].

Laccase can also be used in combination with other modification processes, to create functional groups on ‘inert’ polymers. Nonwoven polypropylene fabrics were pretreated by

argon plasma in the presence of different methacrylate monomers, in order to activate the inert synthetic polymers, followed by laccase-catalyzed grafting of guaiacol sulfonic acid onto the modified surface [117]. In a similar way, cellulose fibers were chemically functionalized with amine groups, that were subsequently coated with enzymatically-synthesized poly(catechol) in the presence of *Trametes villosa* laccase [118]. Phenolic amines were coupled to lignin moieties of wood using the *Trametes hirsuta* laccase [119]. The amine group works as anchor group for further grafting of antifungal molecules via chemical or enzymatic reaction or simply by adsorption, see Figure 8.

Laccases can be used in many different applications, their main feature being that they catalyze oxidative reactions of functionalized aromatic compounds in a mild and environmentally friendly way. Laccases produce radicals that can couple to other aromatic compounds, provided that there are one or more electron-donating groups on the aromatic ring. Considering the structure of poly(ethersulfone) (PES, see Figure 9), it was hypothesized at the start of the work reported in this thesis that this important membrane material would be reactive towards radicals produced by laccase. In this thesis the laccase-catalyzed modification of PES is investigated mostly in relation to protein repellence of membranes, but also in relation to biofouling. The specific aim of the research and a short preview on the contents of the various chapters is given in the next section.

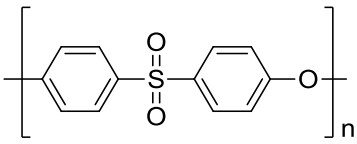


Figure 9. Molecular structure of poly(ethersulfone) (PES).

3. Aim and Outline of the Thesis

From the previous sections it is clear that if a membrane modification method would become available that prevents (protein) adsorption and allows tight control over the (functionality of the) modification layer, this would be of great interest for many different applications. Laccase-catalyzed reactions are known to be versatile and environmentally friendly, and poly(ethersulfone) is a potential reaction partner for the enzyme-generated radicals. In this thesis, these two aspects are brought together: laccase-catalyzed modification of poly(ethersulfone) membranes is investigated in great detail, ranging from a detailed description of the attachment of the polymer and its growth over time, to the membrane performance.

An overview of modification methods for poly(arylsulfone) [*i.e.*, Polysulfone (PSf) and Poly(ethersulfone) (PES)] membranes is presented in ***Chapter two*** with special reference to surface modification. Modification methods are compared on various aspects, such as flux after modification, simplicity, reproducibility, environmental aspects, and cost effectiveness. The enzyme-catalyzed modification method is highlighted as an environmentally benign alternative for other modification methods.

The principle of enzyme-catalyzed modification of PES membranes is proven in ***Chapter three***. Various phenolic acids (enzyme substrates) are investigated under very mild conditions (room temperature, water, nearly neutral pH) using laccase from *Trametes versicolor*. The produced layers are extensively analyzed, both from a chemical and a membrane performance point of view.

In ***Chapter four***, the performance of laccase-catalyzed modified poly(ethersulfone) membranes modified with 4-hydroxybenzoic acid and gallic acid as substrates are evaluated based on *e.g.* grafting yield, flux, and reduction of protein adsorption. Also the effect of the enzyme-catalyzed modification on membrane bulk properties is discussed.

Chapter five gives details on adsorption of BSA, dextrin, and tannin on modified model PES surfaces. Reflectometry is used to follow the adsorbed amount as function of time, and gives information on the adsorption rate, and on the amounts of reversibly and irreversibly adsorbed foulant on the various surfaces. Conclusions are drawn on the importance of various aspects of the modification layer on adsorption.

The effect of modification on suppression of biofouling on model PES surfaces is presented in ***Chapter six***. *Listeria monocytogenes* bacteria are used to evaluate both attachment and biofilm growth under static and dynamic conditions.

A general discussion and an overview of all results is presented in ***Chapter seven***, which is finalized with an outlook for application on industrial scale and future developments.

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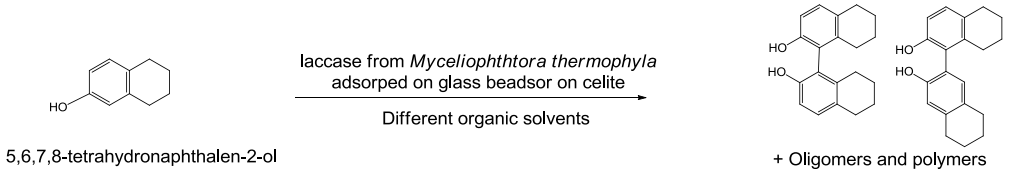
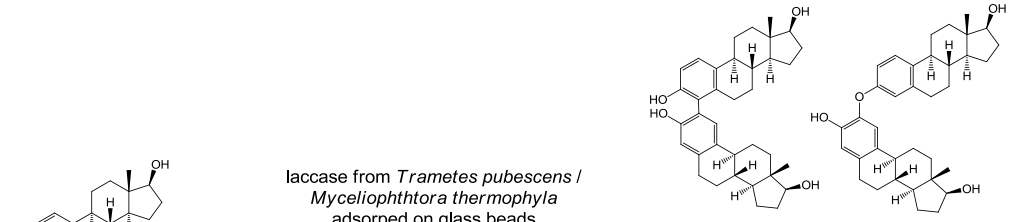
Appendix A. Some monomers and their dimers synthesized by laccase. Ref.



5,6,7,8-tetrahydronaphthalen-2-ol

+ Oligomers and polymers

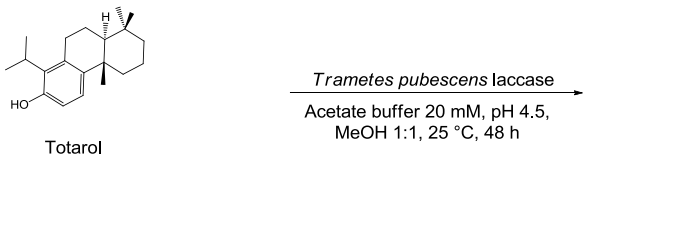
[95]



17-beta estradiol

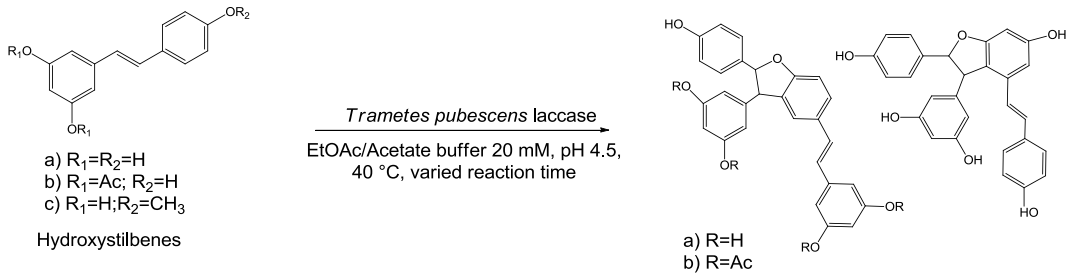
+ unknown C-O dimers, oligomers and polymers

[91,95]



Totarol

[93]

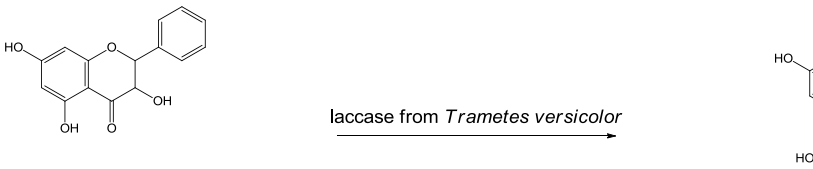


a) R_1=R_2=H
b) R_1=Ac; R_2=H
c) R_1=H;R_2=CH_3

Hydroxystilbenes

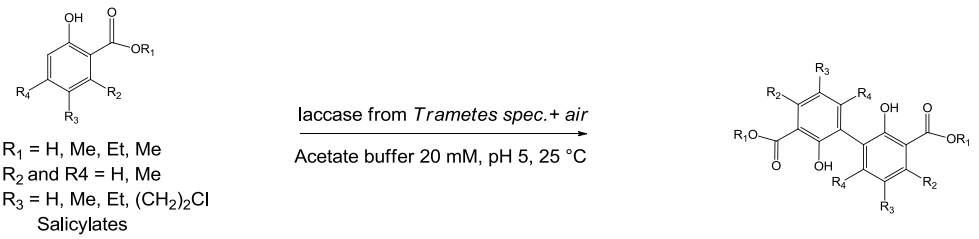
a) R=H
b) R=Ac

[92,94]



Galangin (flavonol)

[100]

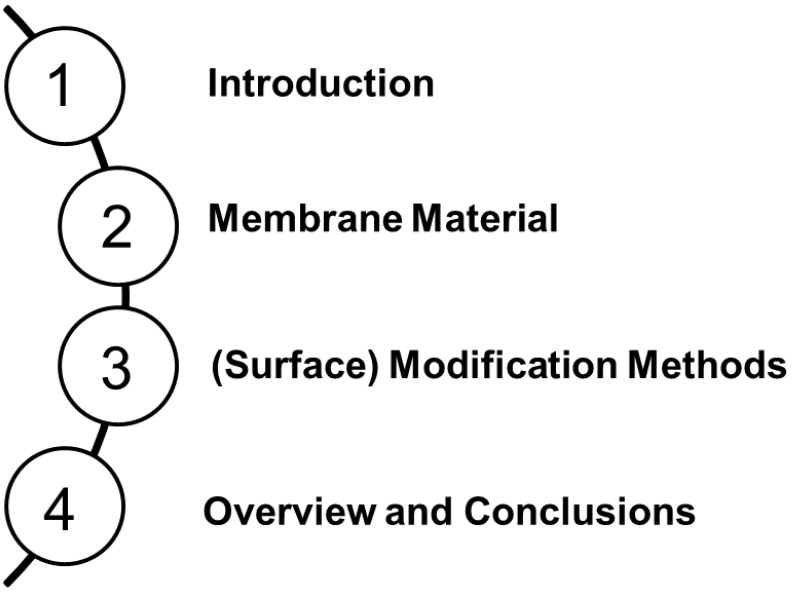


R_1 = H, Me, Et, Me
R_2 and R4 = H, Me
R_3 = H, Me, Et, $(CH_2)_2Cl$
Salicylates

[101]

Chapter Two

Mini-Review on Poly(arylsulfone) Surface Modification



This chapter has been published as:

Modification Methods for Poly(arylsulfone) Membranes: A Mini-Review Focusing on Surface Modification, Norhan Nady, Maurice Franssen, Han Zuilhof, Mohamed Mohy Eldin, Remko Boom, and Karin Schroën, Desalination 275 (2011) 1-9.

Modification Methods for Poly(arylsulfone) Membranes: A Mini-Review Focusing on Surface Modification

Abstract

Surface modification of membranes is thought to be equally important to the membrane industry as membrane material and process development; surface functionalization has already become a key technology, the major aims being performance improvement (flux and selectivity) by reduction of unwanted protein fouling (often considered the first step for biofouling).

Poly(arylsulfone) [*i.e.*, Polysulfone (PSf) and Poly(ethersulfone) (PES)] membranes have been widely used for separation and purification purposes. However, in many cases, nonspecific (protein) adsorption takes place on the membrane surface and in the membrane pores due to the inherent hydrophobic characteristics of poly(arylsulfone). Therefore several (surface) modification techniques for poly(arylsulfone) membranes have been developed. Given the importance of modification methods for these membranes and their operation, we decided to dedicate this mini-review solely to this topic.

The modification methods can be divided into the following main groups: (1) coating, (2) blending, (3) composite, (4) chemical, (5) grafting, or (6) a combination of methods. With all these methods, interesting results were obtained concerning reduction of protein adsorption (see respective sections), although the quantification of improved performance is not straightforward. In the conclusions section, all techniques are compared on various aspects such as flux after modification, simplicity, reproducibility, environmental aspects, and cost effectiveness.

1. Introduction

Polymers are attractive materials for various applications, such as membrane filtration, coatings, composites, microelectronic devices, thin-film technology, biomaterials, and so on. The performance of polymeric materials in many applications relies largely upon the combination of bulk (*e.g.* mechanical) properties in combination with the properties of their surfaces. However, polymers very often do not possess the surface properties needed for these applications. Vice versa, those polymers that have good surface properties frequently do not possess the mechanical properties that are critical for their successful application. Due to this dilemma, (surface) modification of polymers without changing the bulk properties has been a topical aim in research for many years, mostly, because surface modification provides a potentially easier route than *e.g.* polymer blending to obtain new polymer properties. The field is still receiving extensive attention as new applications of polymeric materials emerge rapidly, especially in the fields of biotechnology, bioengineering, and nanotechnology [1,2].

For membrane separation, fouling is a serious problem that can be decreased (or even prevented) using surface modification. Membrane fouling is the accumulation of substances on the membrane surface and/or within the membrane pores, which results in deterioration of membrane performance. The interaction between membrane surfaces and solution components plays an important role in the extent of membrane fouling. In ultrafiltration of *e.g.* protein-containing liquids, fouling occurs due to protein adsorption, denaturation, and aggregation at the membrane solution interface. The importance of hydrophilicity for the prevention of protein adsorption has been shown [3], and has been explained to depend on the fact that the hydrophilic surface attracts so much water that adsorption of proteins is reduced [4] and in some cases, it is even claimed that it is prevented. However, not only the surface hydrophilicity plays a central role for protein repellence but also surface structure has significant impact on membrane anti-fouling performance. In this respect, *e.g.* both steric hindrance and the osmotic effect of hydrated (grafted) polymer branches contribute to resistance against membrane fouling [4-7]. Thus, membrane researchers and manufacturers have, for example, tried to graft different kinds of hydrophilic polymers (with different functional groups) to membranes, or tried to blend polymers to increase hydrophilicity. Besides, sometimes a change in charge density is achieved, which may be beneficial [1].

In membrane manufacturing, surface functionalization of preformed membranes has already become a key technology. The aims of surface modification of a membrane are largely two fold: 1) minimization of undesired interactions (adsorption or adhesion, or in

more general terms membrane fouling) that reduce the performance as described previously; 2) improvement of the selectivity or even the formation of entirely novel separation functions [5]. This can be achieved via the introduction of additional (tailored) interactions (affinity, responsiveness, or catalytic properties). Novel membranes with a high selectivity, *e.g.* for isomers, enantiomers or special biomolecules are in high demand. Consequently, particular attention should be paid to truly molecule-selective separations, *i.e.* advanced nanofiltration and ultrafiltration membranes. In addition, a membrane selectivity that can be switched by an external stimulus or can adapt to the environment/process conditions would be an important feature. Such novel developments may seem futuristic, but it is clear that if such advanced or novel selective membranes were available, they would immediately find applications in many fields such as analytics, screening, membrane reactors, or bio-artificial membrane systems [2].

Many factors need to be considered in the overall process of membrane modification, such as uniformity, reproducibility, stability, process control, and reasonable cost, together with precise control over functional groups, which is a big challenge [1]. Among the surface modification techniques developed to date, surface grafting has emerged as a simple, useful, and versatile approach to improve surface properties of polymers for many applications. Grafting has several advantages: (1) the ability to modify the polymer surface to have distinct properties through the choice of different monomers, (2) the controllable introduction of graft chains with a high density and exact localization to the surface, without affecting the bulk properties, and (3) the long-term chemical stability, which is assured by covalent attachment of graft chains [1,6]. The latter factor contrasts with physically coated polymer chains that can in principle be removed rather easily.

In this chapter, we will limit ourselves to poly(arylsulfone) [more specifically, polysulfone (PSf) and poly(ethersulfone) (PES)], which are very popular membrane materials due to their high performance low cost profile, and for which a great number of modification methods have been published. We will discuss various examples of either ‘grafting-to’ polymerization (coupling polymers to surfaces), or ‘grafting-from’ polymerization (monomers are polymerized using an initiation reaction on the surface) [5-7], together with other methods that are used for membrane modification. We will give illustrative examples on how the membrane (performance) is improved, although it should be noted that frequently more than one membrane parameter is influenced, which not all may be advantageous. We will mainly focus on reduction of protein adsorption of poly(arylsulfone) membranes, provided that the flux is not influenced dramatically by the modification layer. In the discussion section, the

methods are compared and rated on their applicability for modification of poly(arylsulfone) membranes.

2. Membrane Material

Nowadays, poly(ethersulfone) (PES, see Figure 1) is the most popular material for ultrafiltration and microfiltration membrane manufacture. This material provides robust membranes due to its structural and chemical stability. Further, high flux and reasonable cost compared to other membrane materials, add to the popularity of this polymer. Unfortunately, PES is a hydrophobic material, with a relatively low surface energy and high water contact angle, and membranes made from such material are more vulnerable to adsorptive fouling.

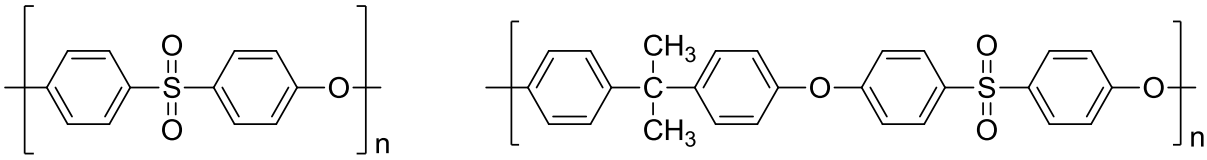


Figure 1. Molecular structures of PES (left) and PSf (right).

In order to capitalize on the usefulness of PES membranes in filtration operations, many studies have investigated (surface) modification of this material to make it polar and less hydrophobic. Excellent results have been achieved by using surface modification techniques such as photo-induced grafting to improve PES membrane wettability. Also, blending the PES with a hydrophilic polymer to get new material with more hydrophilic surface properties has been reported.

This chapter provides a comprehensive overview on potential (surface) modification techniques for PES membranes and polysulfone membranes (PSf, see Figure 1), which are very comparable in structure [*i.e.*, poly(arylsulfone) membranes]. Several modification methods for commercially available poly(arylsulfone) membranes have been developed. These methods can be divided into six main groups: (1) coating, (2) blending, (3) composite, (4) chemical, and (5) grafting. In addition, (6) combined methods are discussed. The methods are first discussed individually; in the section thereafter they are compared.

3. (Surface) Modification Methods

3.1. Coating (Thin Film Composites)

Coating is a method wherein the coating material(s) forms a thin layer that non-covalently adheres to the substrate. Coating methods can be divided into five techniques: coating of a

hydrophilic thin layer by physical adsorption [8-10], possibly followed by curing with heat [11,12], coating with a monolayer using Langmuir-Blodgett or analogous techniques [13], deposition from a glow discharge plasma [14], and casting or extrusion of two polymer solutions by simultaneous spinning using *e.g.* a triple orifice spinneret. In the latter technique, using different solvents for each polymer solution facilitates adhesion between the upper coating layer and the base polymer [15,16].

Here we give some examples of coated membranes to illustrate the versatility of the technique. Charged membranes were prepared by coating PES ultrafiltration membranes with sulphonated poly(2,6-dimethyl-1,4-phenylene oxide) [17]. PSf membranes were dipped in methyl methacrylate-based comb polymers with short oligoethylene glycol side chains that provide the membrane with long-term, bio-repellant surfaces; cell-lysate flux recovery increased from 47% for unmodified PSf membranes to 94% for the coated membrane after a five-cycle filtration-washing process [18]. It is claimed that this is caused by the hydrophilic polyethylene oxide (PEO) groups on the surface. The effect of TiO_2 nanoparticle insertion into the PSf membrane to increase its hydrophilicity was tested by dipping a neat PSf membrane surface into a 1% TiO_2 aqueous suspension and pressurizing it at 400 kPa. The TiO_2-deposited membrane showed a higher fouling mitigation effect compared to a TiO_2-entrapped membrane (*i.e.*, TiO_2 nanoparticles mixed with PES, see composite section). The initial flux loss due to fouling by adsorption in the beginning of filtration decreased from 60 to 15% relative to the original fluxes (22% in case of TiO_2-entrapped membrane). This could be attributed to the higher number of TiO_2 nanoparticles (as deduced from SEM images that were used to distinguish between deposited and entrapped particles) deposited on the membrane surface through coating; the degree of fouling mitigation is linked to the surface area of exposed TiO_2 nanoparticles [19]. In a quite different example for preparing a nonporous membrane suitable for gas separation (*i.e.*, separation depends on different solubility and diffusivity of different gases in the polymer of the separation layer), 6FDA-durene-1,3-phenylenediamine (50:50) copolyimide (see Figure 2) was prepared and was used to form the outer, asymmetric separating layer of fluoropolyimide/polyethersulfone dual-layer hollow fiber membranes [20]. In this system, the actual separation layer was deposited on the PES support by using a newly designed dual-layer spinneret that allowed depositing a very uniform thin (10 μm) separating layer by co-extrusion and dry-jet wet-spinning phase inversion. This thin film showed a high O_2/N_2 selectivity value (4.6). This new design could be valuable in laminating a thin layer of new polymers or composite on poly(arysulfone) supports, to be used in separation of fluids.

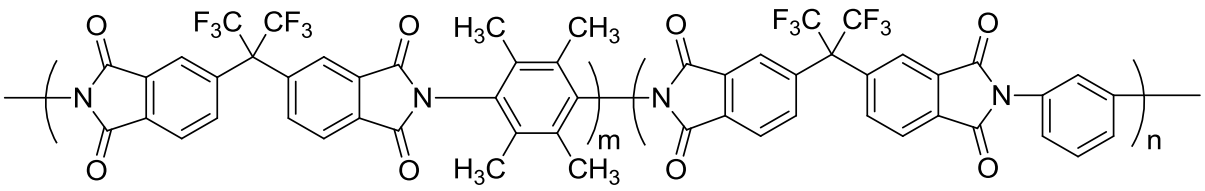


Figure 2. Structure of 6FDA-durene-1,3-phenylenediamine copolyimide.

Recently, thermal cross-linking of poly(ethylene glycol) diacrylate on PES membranes was published, using trimethylolpropane trimethylacrylate as an accelerator [21]. The best membrane performance was achieved at 150 μg·cm^{-2} mass gain, which corresponds to approximately 25% less flux reduction, and this is attributed to the presence of the hydrophilic polyethylene glycol (PEG) groups. Also, PES membranes were coated by the strong chelating agent diethylene triamine pentaacetic acid [22]. The modified membranes changed from ultra- to nanofiltration membranes, with which 93% and 100% removal of heavy metals and suspended solid/total dissolved solid, respectively, could be achieved. It is clear that various highly advantageous effects can be achieved through this coating, although the stability of the coating during separation processes is always a point of concern.

3.2. Blending

Blending is a process in which two (or more) polymers are physically mixed to obtain the required properties. Blend polymer membranes based on PES have been successfully prepared in combination with *e.g.* PEG [23], poly(vinylpyrrolidone) [24,25], cellulose acetate [26], cellulose acetate phthalate [27], soybean phosphatidylcholine [28], or tetronic1307 [29]. Although compatible polymers have been identified, and membranes prepared from them, in general it has to be mentioned that in depth investigation and optimization of the membrane formation process is needed, since it will differ considerably from the formation process for the basic polymer. Further, also other properties such as the mechanical strength have to be evaluated since these are also expected to differ from the original. Unfortunately, this characteristic is hardly mentioned in literature. Alternatively, surface modifying macromolecules (SMM's) - synthesized from methylenebis-(phenyl diisocyanate), poly(propylene diol), and a fluoroalcohol - have been used [30]. Besides, the use of branched amphiphilic copolymers (P123-b-PEG) [31] and of an amphiphilic comb-copolymer with polystyrene as hydrophobic part and PEG [32] has also been reported. In the latter case, the hydrophilic PEG segments spontaneously segregated to the membrane surface during immersion precipitation, which increased hydrophilicity and reduced protein adsorption from

6.8 to 0.5 μg·cm^{-2}, whereas only a slight change in permeation properties was observed. Comparable results were found for PSf-based blended membranes with amphiphilic copolymers having PSf backbones and PEG side chains, (PSf-g-PEG) [33]. These membranes exhibited good mechanical characteristics, and remarkably reduced protein adsorption (about 72% reduction in protein adsorption with 10 wt% PSf-g-PEG blending).

Recently, amphiphilic copolymers such as phosphorylcholine copolymer [34] (*i.e.*, synthesized copolymer composed of 2-methacryloyloxyethylphosphorylcholine (MPC) and n-butyl methacrylate (BMA)) were investigated. Blending of this MPC-BMA copolymer with PES membranes reduced the contact angle from 71° to 39°, and bovine serum albumin (BSA) adsorption from 65 to 10.6 μg·cm^{-2}, which the authors attributed to increased hydrophilicity. Although nice results were obtained with blending of amphiphilic copolymers with PES, only few of theses amphiphilic copolymers such as tetronic [29] have been synthesized on commercial scale.

3.3. Composite

A composite is a material made from two or more materials with different physical or chemical properties which remain separate and distinct on a macroscopic level within the finished structure. N,O-carboxymethyl chitosan/poly(ethersulfone) (CM-CS/PES) [35] composite membranes were prepared by immersing PES microfiltration membranes into CM-CS solutions and cross-linking with glutaraldehyde. Streaming potential measurements indicate that CM-CS/PES composite membranes possess a weak positive charge at low pH and a rather strong negative charge at high pH [36]. Therefore, the negative electrostatic repulsion interactions between membrane and protein molecules at pH 6-8 (*i.e.*, above BSA isoelectric point) are stronger than the positive electrostatic repulsion interactions at pH 3-4 (*i.e.*, below BSA isoelectric point). Under acidic conditions, relatively high adsorption levels occur, also caused by denaturation and aggregation of the protein below its isoelectric point. This gives the CM-CS/PES composite membranes a dual functionality; they resist protein fouling at high pH, and separate proteins by adsorption at low pH, which can subsequently be recovered by increasing the pH.

Sulfonated poly(ether-ethersulfone)-poly(ethersulfone) (SPEES-PES) and sulfonated poly(ether-ethersulfone)-poly(ethersulfone)/Poly (vinyl chloride) (SPEES-PES/PVC) [37] composites were used to measure glucose and hydrogen peroxide permselectivity in amperometric biosensors. Also, highly charged cation-permeable composite membranes were prepared from blends of sulfonated PES with sulfonated poly(ether-ether-ketone) [38].

TiO_2 nanoparticles were added to the polymer solution and then TiO_2-entrapped PSf membranes were prepared by phase inversion [19]. These membranes showed less flux decline (38%) compared to neat PSf membranes (85%). Also, PES-TiO_2 composite membranes (4% wt) showed better flux behavior (29% higher) compared to PES membranes [39]. On the other hand, the included TiO_2 nanoparticles resulted in improvements in mechanical properties of PES membrane by increasing the breaking strength from 3.2 to 4.1 MPa while decreasing the elongation ratio from 16 to 12%. Fouling mitigation increased with nanoparticle content, but it reached a limit above which fouling mitigation was not improved. The TiO_2 nanoparticle acts mainly on hydrophobic substances, suggesting a possible use as a new anti-fouling component in composite membranes [40]. Using Al_2O_3 instead of TiO_2 and at much lower concentration [10 times lower] resulted in reduction in cake formation from 82% to 18%. The flux loss during operation was diminished by over 10% [41].

Similar to the case of blending of amphiphilic copolymers - the range of components that are suitable for composite formation and are ready available and have been synthesized on large scale is limited.

3.4. Chemical

For chemical modification, the membrane material is treated with modifying agents to introduce various functional groups on the membrane surface. For example, [$-CH_2CH_2CH_2SO_3^-$] [42,43] groups have been coupled onto the surfaces of PSf hollow fibers using the reaction of PSf, propane sultone, and Friedel-Crafts catalysts. The resulting membranes were claimed to show excellent anti-adsorption behavior. Also, a surface reaction of PSf hollow fibers with propylene oxide and a Friedel-Crafts catalyst was carried out, and a hydrophilic surface without charged segments ("hydroxyl" type; $-CH(CH_3)CH_2OH$) [44,45] was obtained. The membranes were tested by ultrafiltration of a mix of BSA and γ-globulin. It was found that BSA is concentrated in the retentate and γ-globulin is concentrated in the permeate when a modified membrane with $-CH(CH_3)CH_2OH$ segments is used, while the unmodified membranes cannot separate the proteins. The ultrafiltration of the mixture at pH 9 (BSA and γ-globulin have the same net negative charge) suggested that the separation mechanism is not due to a sieving effect or to charge repulsion but resulted from the balance of hydrophilic and hydrophobic segments on the surface of the modified membranes.

In addition, sulfonation, chloromethylation, aminomethylation, and lithiation reactions were applied to PSf membranes [46-48]. The main challenge for modification by chemical treatment of commercial membranes is that the modification agent may partly block the pores

of the membranes. Even if the modified membranes are less prone to fouling, the total flux after modification is generally smaller than before modification. In some cases, chemical modification during membrane formation is preferred, since it seems to compromise the flux less [49].

3.5. Grafting

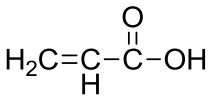
Grafting is a method wherein monomers are covalently bonded onto the membrane. Some examples of monomers used for PES modification are shown in Figure 3. The techniques to initiate grafting are: (i) chemical, (ii) photochemical and/or via high-energy radiation, (iii) the use of a plasma, and (iv) enzymatic. The choice for a specific grafting technique depends on the chemical structure of the membrane and the desired characteristics after surface modification.

3.5.1. Chemical Initiation Technique

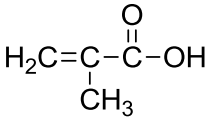
In chemical grafting, free radicals are produced that are transferred to the substrate to initiate polymerization and form graft co-polymers. A few studies showed that redox initiation-grafting could be successfully applied to PES ultrafiltration membranes [50,51]. For these, peroxydisulfate and metabisulfite oxidizing agents have been used to initiate free-radical polymerization grafting of methacrylic acid, polyethyleneglycol-methacrylate, and sulfopropylmethacrylate in aqueous solution at ambient temperature. In general, this technique is simple and cheap, leading to membranes that are claimed to be less sensitive to fouling due to the presence of the hydrophilic grafted monomers, but it is a harsh treatment.

3.5.2. Photochemical and Radiation Initiation Techniques

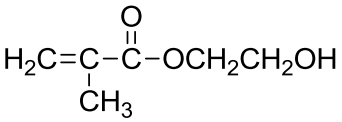
When a chromophore on a macromolecule absorbs light, the molecule is brought in an exited state, and one or more chemical bonds may dissociate into radicals that can act as initiators for the grafting progress. Radicals that are generated in this manner on the membrane surface can react with the monomer to form the grafted co-polymer. If the absorption of light does not lead to the formation of free radical sites, this can be promoted by addition of photosensitizers that form radicals, which in turn abstract hydrogen atoms from the base polymer surface and produce the radical sites required for grafting [11].



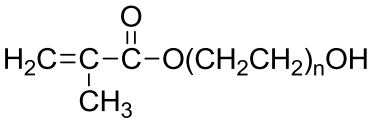
acrylic acid



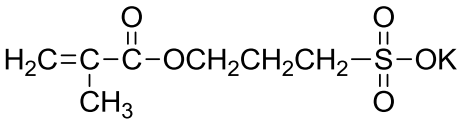
methacrylic acid



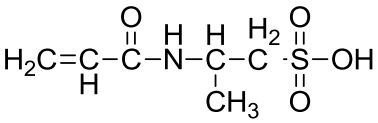
2-hydroxyethyl methacrylate



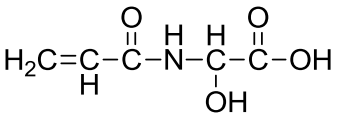
poly(ethylene glycol) methacrylate



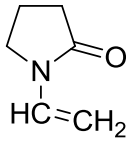
3-sulphopropyl methacrylate potassium salt



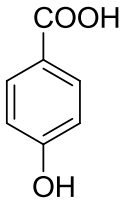
2-acrylamido-2-methyl-1-propanesulphonic acid



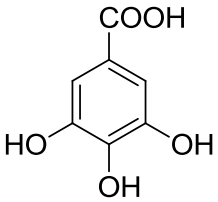
2-acrylamido glycolic acid



N-vinyl-2-pyrrolidone



4-hydroxybenzoic acid



gallic acid

Figure 3. Chemical structure of some monomers used to modify PES membranes.

The irradiation of macromolecules can cause homolytic fission and thus forms free radicals on the membrane. UV irradiation and UV-assisted graft polymerization are techniques that can selectively alter membrane surface properties without affecting the bulk polymer. UV-assisted graft polymerization modifies the membrane surface by grafting polymer chains onto the surface and in the pores. UV irradiation can cross-link polymer chains and cleave polymer bonds, therewith forming functional groups such as hydroxyls, carbonyls, or carboxylic acids

on the surface. Initial attempts to carry out the graft modification of poly(arylsulfone) membranes were conducted in the presence of benzophenone as a photosensitizer [52]. However, it was soon discovered that all poly(arylsulfone) membranes are intrinsically photosensitive and generate free radicals upon irradiation with 254 nm UV light [53-55]. The UV irradiation should be carefully used because it leads to severe degradation of the pore structure with loss of membrane function, which needs to be partially compensated by grafted polymer. This technique was used to graft several hydrophilic monomers (N-vinyl-2-pyrrolidinone, N-vinylcaprolactam, and N-vinylformamide) onto 10 kDa PES ultrafiltration membranes, and their fouling during BSA filtration was compared with that of an unmodified membrane. Membranes modified with N-vinyl-2-pyrrolidinone (25% increase in hydrophilicity) exhibited the best combination of low fouling (50% decrease in BSA fouling) and high flux, although membrane permeability was significantly decreased because the grafted polymer chains blocked the membrane pores (over 25% reduction in flux due to modification) [56].

UV-assisted graft polymerization of the same monomer (N-vinyl-2-pyrrolidinone) onto 50 kDa PES membranes created highly wettable PES membranes with high fouling resistance compared to the base membrane [57-59]. Two methods were used: dip modification (irradiation after dipping in monomer solution) and immersion modification (irradiation in monomer solution). The irreversible flux decrease due to adsorptive fouling, *i.e.* the permanent flux drop after water cleaning with respect to the initial buffer flux, was reduced significantly, from 42% for the base membrane to 9% for the modified membrane. The immersion technique created the best membranes for applications in which high protein retention is required, while the dip-modified membranes performed best for applications in which high protein transmission is preferred. When comparing the dip and immersion method it becomes clear that the dip method seems more suitable for industrial applications. It requires less monomer, it can more easily be adapted to a continuous process, and is easier to control.

UV-assisted graft polymerization was used for three hydrophilic monomers, N-vinyl-2-pyrrolidinone, 2-acrylamidoglycolic acid monohydrate, and 2-acrylamido-2-methyl-1-propanesulfonic acid, on PES and PSf membranes of 50 kDa. The ultrafiltration membranes were modified using the dip method with 300 nm wavelength lamps [60]. Four conditions were found to give superior filtration performance: high monomer concentrations (5 wt%), low irradiation energy (<65 $mJ \cdot cm^{-2}$ for PES and <130 $mJ.cm^{-2}$ for PSf), low degree of grafting (*i.e.*, DG < 0.53), and intermediate wettability (contact angle between 35^{o} - 42^{o}).

Most probably, under these conditions, the grafted polymer chains are placed in such a way that they extend from the surface, therewith preventing protein penetration. Interestingly, it was found that PES is much more sensitive to UV-assisted graft polymerization than PSf, and thus, requires far less energy to attain a desired degree of grafting. Surface modification of PES ultrafiltration membranes via simultaneous photografting polymerization has been successfully done to prepare low-fouling UF membranes (≥ 300 nm wavelength lamps). The hydrophilic monomers that were used were poly(ethylene glycol) methacrylate [61], N-2-vinyl pyrrolidinone, 2-hydroxyethyl methacrylate, acrylic acid, 2-acrylamidoglycolic acid, 3-sulfopropyl methacrylate, 2-acrylamido-2-methyl-1-propanesulfonic acid [62,63], quaternary 2-dimethylamino-ethylmethacrylate [64]. Moreover, polymers such as poly(vinyl alcohol), polyethylene glycol, and chitosan [65] have been photografted onto PES membranes. Membrane permeability increased by 50% for UV dip-modified membranes in the presence of a low concentration (10 mM) of chain transfer agent (2-mercaptoethanol) and by 20-200% (with severe reduction in membrane rejection) when high (50 mM) 2-mercaptoethanol concentrations were used [66].

Radicals can also be formed by electromagnetic radiation of a shorter wavelength (*i.e.*, gamma irradiation) [38,67]. Free radical grafting initiated by radiation proceeds in three different ways, which are pre-irradiation, peroxidation, and mutual irradiation. In pre-irradiation grafting, the membrane backbone is first irradiated in vacuum or in the presence of an inert gas to form free radicals. Then, the irradiated membrane is treated with the monomer, in liquid or vapor state. In peroxidation grafting, the membrane is subjected to radiation in the presence of air or oxygen to form hydroperoxides or diperoxides. The stable peroxy products are then treated with the monomer at higher temperature, where the peroxides undergo decomposition to radicals and initiate grafting. In mutual irradiation, the membrane and the monomers are irradiated simultaneously, to form free radicals that are subsequently grafted to the surface. Radiation grafting can also proceed through an ionic mode [68].

Resistance of poly(arylsulphone) resins to γ-irradiation in vacuum and air at various temperatures has been studied [67,69]; both cross-linking and chain scission occur when PSf and PES were subjected to γ-irradiation at 30 °C. Cross-linking is predominant for irradiation under vacuum, whereas for irradiation in air scission predominates over cross-linking. Asymmetric PES ultrafiltration membranes have been modified with acrylic acid or acrylamide [70], and at certain combinations of experimental parameters, water flux, and solute retention were improved compared with the untreated membrane.

3.5.3. Plasma Initiation Technique

Given enough energy, any gas can be excited into the plasma state, which is a mixture of ions, electrons, excited species, and free radicals. Plasma surface treatment usually refers to a plasma reaction that either results in modification of the molecular structure of the surface, or atomic substitution. Plasma treatment is a useful tool in the modification of surface properties. Currently, more and more attention is being given to its applications in membrane separation science. The accelerated electrons from the plasma have sufficient energy to induce cleavage of the chemical bonds in the membrane structure and to form macromolecule radicals, which subsequently initiate graft co-polymerization [71]. Plasma treatment can be done by either regular plasma treatment [72,73], or plasma graft co-polymerization (PGC) [74]. Low temperature plasma techniques, which are very surface selective, have been used to modify various types of membranes, specifically to reduce protein-surface attractive interaction. For example, simple inert gas [75,76], nitrogen [73], or oxygen [72] plasmas have been used to increase the surface hydrophilicity of membranes [14,77], and ammonia plasmas have successfully yielded functionalized PSf membranes [78]. Also a water plasma treatment that renders asymmetric PSf membranes permanently hydrophilic has been reported [79], and this technique was also successfully applied to PES membranes and, to a lesser extent, polyethylene membranes [77,80]. Further, Ar-plasma treatment followed by graft copolymerization with acrylamide in the vapor phase was used to make PES membranes highly hydrophilic [74]. The grafting yield for polyacrylamide on the membrane surfaces increased nearly linearly with the Ar-plasma pretreatment time, with grafting yields (GY) higher than 100 $\mu g \cdot cm^{-2}$. The membranes obtained a permanent hydrophilicity (almost no change in contact angle after one year), and BSA adsorption was reduced to less than half that of the control membrane (306 to 148 $\mu g \cdot cm^{-2}$). Although, plasma treatment (without grafting) is often claimed to increase hydrophilicity (attributed to structural rearrangement of polymer chains with decrease in surface energy [76]), and therewith protein repellence, mostly this effect tends to be temporary, which could imply that the treatment has to be repeated.

3.5.4. Enzymatic Initiation Technique

The enzymatic grafting method is quite new. The principle involved is that an enzyme initiates the chemical/electrochemical grafting reaction [71]. This method employs enzymes to convert the substrate (monomer, oligomer or polymer chains) into a reactive free radical(s), which undergoes subsequent non-enzymatic reaction with the membrane [81-86]. There are several potential advantages for the use of enzymes in membrane modification. With respect

to health and safety, enzymes offer the potential of eliminating the need for (and hazards associated with) reactive reagents (and solvents). A potential environmental benefit for using enzymes is that their selectivity may be exploited to eliminate the need for wasteful protection and deprotection steps. Finally, enzyme specificity may offer the potential for precisely modifying macromolecular structure to better control polymer function [83]. Some polymers that were successfully modified with enzymes are mentioned here briefly.

Enzymatic grafting of chitosan resins and films using tyrosinase and chlorogenic acid [83], 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, and hydrocaffeic acid phenol derivatives [84,85] has been reported. The main target for chitosan enzyme-catalyzed grafting is to alter the surface and rheological properties, under basic conditions, and cationic dye-adsorption properties. Reactions were conducted under heterogeneous conditions using chitosan films, and under homogeneous conditions using aqueous methanolic mixtures capable of dissolving both substrates and chitosan [84]. Tyrosinase was shown to convert the substrate into a reactive *o*-quinone, which undergoes a subsequent non-enzymatic reaction with chitosan. Tyrosinase has a broad substrate range for phenols such as poly(4-hydroxystyrene), 4-tert-butylcatechol, *p*-hydroxyphenoxyacetic acid and *p*-cresol [81,82]. In addition to reacting with a range of monomeric phenols, tyrosinase is known to react with oligomeric and polymeric substrates [86], therewith indicating the versatility that enzyme-catalyzed modification could yield.

Very recently, we published an enzyme based method for grafting of PES membranes [87]. This modification method uses laccase from *Trametes versicolor* to create free radicals, and graft phenolic acid monomers (*e.g.*, gallic acid or 4-hydroxybenzoic acid) to the membrane. The modified membranes have high fluxes and in some cases excellent protein repellent properties, but a clear relation between the grafted layer and its effectiveness against protein adsorption has not been established yet. What is clear, is that this modification method is very mild and environmentally benign compared to the more traditional methods; it can be carried out at room temperature, and uses only oxygen and water, and no toxic chemicals.

3.6. Combined Methods

Recently, combined techniques were presented for PES membranes [88,89], in which the membrane was blended with a copolymer of acrylonitrile and acrylic acid, and subsequently grafted with bovine serum albumin [88]. Sulfonic acid groups were generated on the PES membrane surfaces by chemical sulfonation, followed by dipping the membranes into a TiO_2 solution [89,90]. This modification (8 % wt. TiO_2 deposited) reduces the loss of flux due to

fouling from 80 to 65%. Another combined modification was carried out by blending PES with polyimide and treatment with diethanolamine to introduce –OH groups on the membrane surface, followed by dipping in TiO_2 colloidal solution, and irradiation with UV light [91]. Combined modification sequences lead to lower fouling of the membrane. The results as such are interesting, although the complexity of the technique could prove to be a major hurdle.

4. Overview of Modification Methods and Conclusions

An overall comparison between the different surface modification methods is presented in Table 1, which is our interpretation of the presented results in literature. To the criteria that have been mentioned thus far has now been added 'cost effectiveness', a factor that depends on the costs of chemicals and equipment used. Note that it is not always straightforward to interpret and compare results, because many parameters may be influenced simultaneously by one modification method; here we only attempt to give a general impression.

All the surface modification methods mentioned earlier allow modification without affecting the bulk properties too much when appropriate conditions are selected; mostly the flux is similar to the base membrane or slightly lower (as indicated by the yellow circles in Table 1).

It is well known that modification by creating a chemical bond (*i.e.,* covalent bonding) is more stable than physical adhesion (*e.g.,* coating). Complete and seemingly permanent hydrophilic modification of poly(arylsulfone) membranes is achieved by blending and photoinduced grafting, although it should be mentioned that protein adsorption is reduced at the produced hydrophilic surfaces, but never completely prevented. Chemical treatment usually employs harsh treatment; often it may lead to undesirable surface changes and contamination, and may not be the best choice in environmental terms. Plasma treatment is probably one of the most versatile poly(arylsulfone) membrane surface treatment techniques. However, its high costs and technical complexity remain drawbacks for large-scale use. Enzyme-catalyzed grafting of poly(ethersulfone) membranes, which has just been reported, is in this respect a method that could be an environmentally benign alternative for other poly(arylsulfone) modification methods, but is in need of further development. Combination of two or three modification techniques is complex in terms of cost effectiveness and environmental drawbacks, but could lead to multi-functional membranes that are of great interest for 'membranes of the future'. Such membranes may need more functions than 'only' providing a selective barrier with high performance (flux and stability). It is expected that membrane properties can be tuned for specific applications through the discussed methods,

although they still need to be developed further in such a way that they allow even better and more environmentally friendly control over modification. To be complete, it should be noted that all mentioned methods influence membrane smoothness/roughness. However, since its effect on protein repellence and flux is still heavily debated in literature as illustrated in [92], we will not consider it further.

Table 1. Advantages and disadvantages of modification methods.

No.	Modification method	Flux after modification	Simplicity/ Versatility	Reproducibility	Environmental aspects	Cost effectiveness
1	Coating	🔴	🟡	🟡	🟡	🟡
2	Blending	🟡	🟢	🟡	🟡	🟡
3	Composite	🟡	🟡	🔴	🟡	🟡
4	Chemical	🔴	🟡	🔴	🔴	🟡
5	Grafting initiated by:					
	Chemical	🟡	🟡	🟡	🔴	🟡
	Photochemical	🟡	🟡	🟡	🟡	🟡
	Radiation	🟡	🔴	🔴	🔴	🔴
	Plasma	🟡	🔴	🔴	🟡	🔴
	Enzymatic	🟡	🟢	🟡	🟢	🟢
6	Combined methods	🔴	🔴	🟡	🔴	🔴

🟢 Excellent, 🟡 High, 🔴 Low

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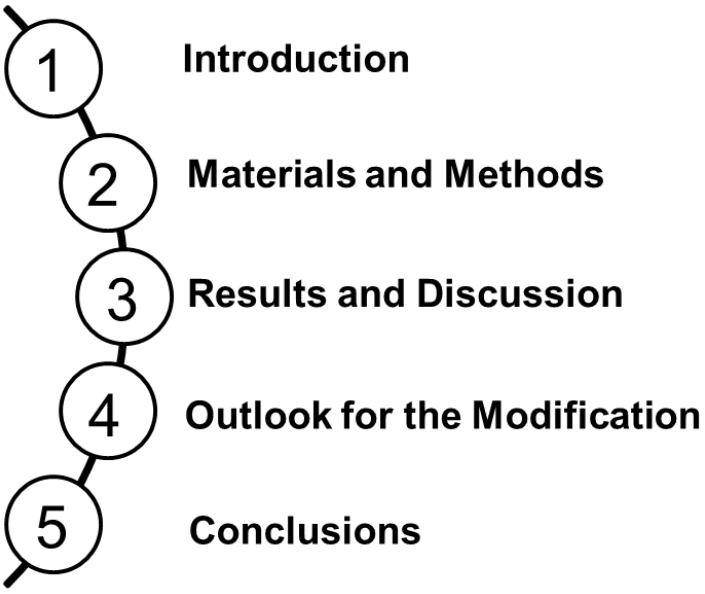
Chapter Three

Enzyme-Catalyzed Modification of PES Membranes. Proof of Principle



This chapter has been published as:

Mild and Highly Flexible Enzyme-Catalyzed Modification of Poly(ethersulfone) Membranes, Norhan Nady, Karin Schroën, Maurice Franssen, Barend van Lagen, Sukumaran Murali, Mohamed Mohy Eldin, Remko Boom, and Han Zuilhof, ACS Appl. Mater. Interfaces 3 (2011) 801-810.

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

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 *e.g.* 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 nuclear magnetic resonance (NMR). In 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 and NMR. 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.

1. 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.

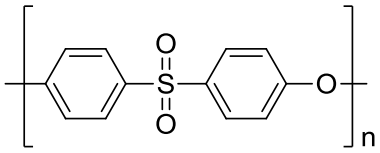


Figure 1. Molecular structure of PES.

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 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 de-polymerization 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 towards 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].

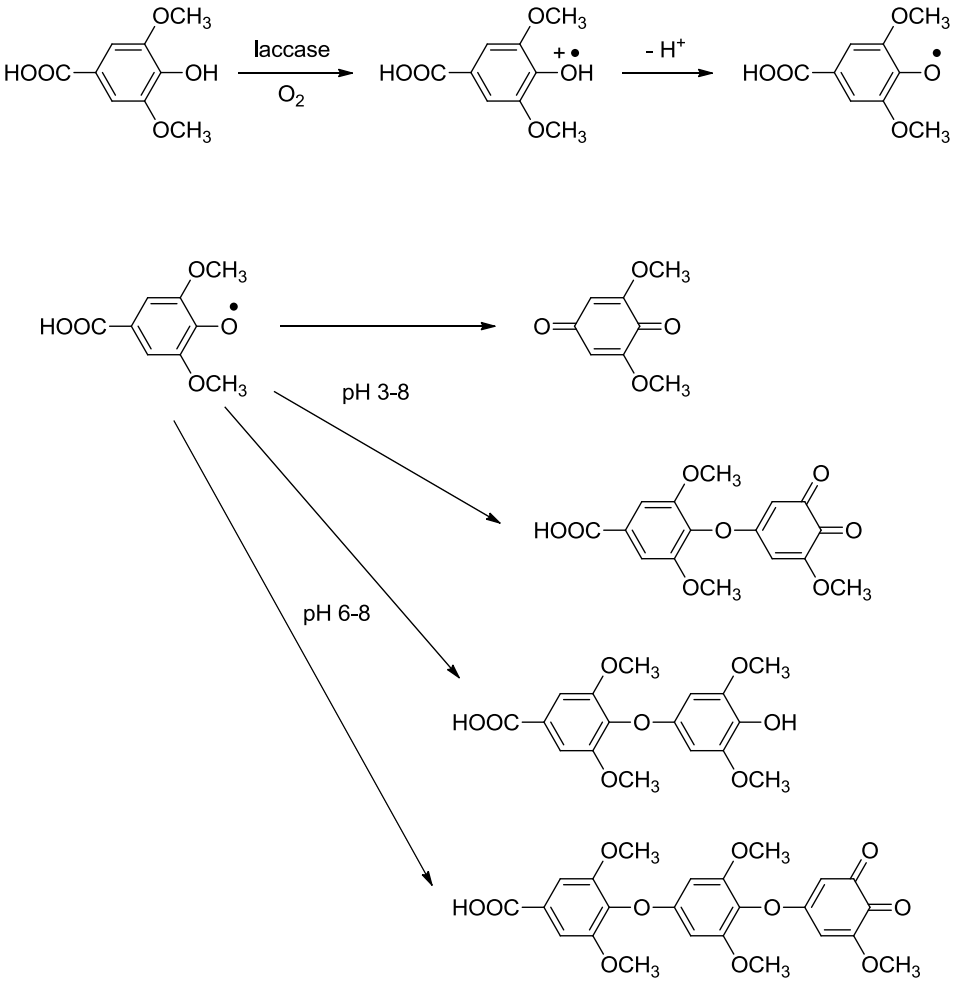


Figure 2. Transformation products from syringic acid (upper left) by laccase of *Rhizoctonia praticola* and *Trametes versicolor* at different pHs [21].

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].

This chapter 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 towards 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 nuclear magnetic resonance (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.

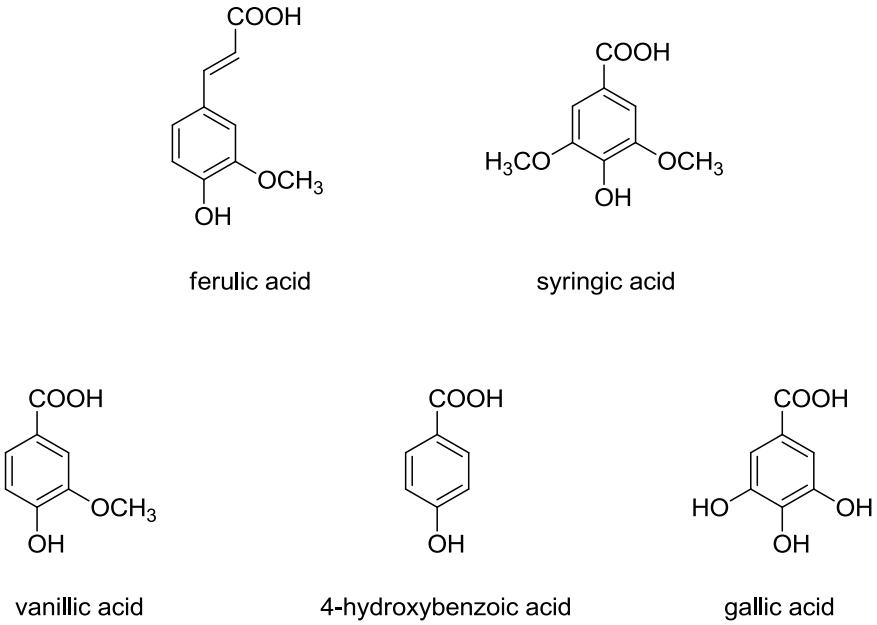


Figure 3. Phenolic monomers (substrates) used in this study.

2. Materials and Methods

2.1. 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-sulphonic 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 $ml \cdot cm^{-2} \cdot min^{-1}$ at $\Delta P = 1$ bar). All chemicals were used as received. Milli-Q water was used in all experiments.

2.2. Laccase Assay

Laccase activity was determined with 2,2′-azobis(3-ethylbenzothiazoline-6-sulphonic 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 $U \cdot ml^{-1}$ laccase. Oxidation of ABTS is monitored by following the increase in absorbance at 420 nm ($\varepsilon = 36{,}000\ M^{-1} \cdot cm^{-1}$) [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 $U \cdot ml^{-1}$ laccase. Oxidation of catechol is monitored by following the increase in absorbance at 400 nm (ε = 26,000 $M^{-1} \cdot cm^{-1}$) [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 & V_{max} = $455 \cdot 10^{-3}$ $mM \cdot min^{-1}$, and for catechol K_m = 8.4 mM & V_{max} = $50.3 \cdot 10^{-3}$ $mM \cdot min^{-1}$]. These values are comparable to the values found for fungal laccases [32].

2.3. Color Measurements

The CIELAB coordinates for the modified membranes were measured with a Color Flex (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 appear more intense than the same color with less saturation. ΔE* is the degree of total color change, which is calculated from $[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$.

2.4. 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.

2.5. 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 Auto-seagull is working with a single angle (range from 85° till 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.

2.6. 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.

2.7. 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 ºC and 300 rpm.

The pure water flux is calculated with Equation 1, in which, J_w = water flux ($m^3 \cdot m^{-2} \cdot s^{-1}$), Q = quantity of permeate collected (m^3), Δt = sampling time (s), and A = the membrane area (m^2). 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].

$$J_w = \frac{Q}{\Delta t \cdot A} \qquad \text{(Eqn. 1)}$$

2.8. 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 modification time, they were removed from the liquid. Ferulic acid, vanillic acid, syringic acid, 4-hydroxybenzoic acid, and gallic 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 $U \cdot ml^{-1}$. 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.

2.9. 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.

2.10. Nuclear Magnetic Resonance (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^{-1}, sodium acetate buffer pH 5, 25°C, modification time 24 h) was completely dissolved in DMF-d7 and subjected to NMR analysis.

2.11. Blank Experiments

2.11.1. Enzyme and Substrate Adsorption Tests

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).

2.11.2. 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 re-adjusting 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.

2.12. 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 $<S^2>$ values were below 0.751 in all cases.

3. 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 $U \cdot ml^{-1}$ enzyme concentration, 24 h modification time, 24 ± 1 ºC, pH = 5 (0.1 M sodium acetate buffer); these conditions are used throughout this chapter 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.

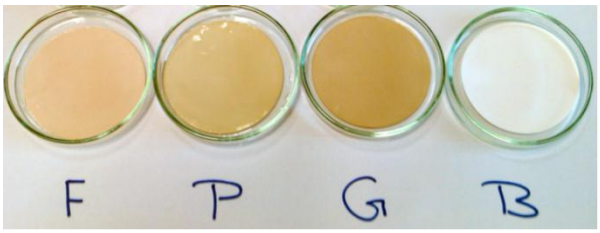


Figure 4. Color changes of modified circular membranes (wet membranes) after enzyme-catalyzed modification with ferulic acid (**F**), 4-hydroxybenzoic acid (**P**), gallic acid (**G**), (**B**) is the blank membrane.

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 $mg \cdot m^{-2}$ 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 towards 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 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 of 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 hours, 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.

Table 1. Lightness, degree of total color change, and color saturation response of modified PES membranes as determined by CIELAB measurements.

	Modification conditions		**ΔL***	**ΔE***	**ΔC***
Substrate	Substrate concentration (mM)	Modification time (h)			
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
	10.0	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
	10.0	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
	10.0	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*

Typical error limits: ± 0.1 for lightness (ΔL*) and color saturation (ΔC*) and ± 0.2 for the degree of total color change (ΔE*)

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, XPS analysis was carried out. 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 (-**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 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).

Table 2. Analysis of XPS spectra of modified PES membranes. Apart from the standard modification time of 24 h, for gallic acid also 2 h modification was used.

	C_{1s}	C_{1s}	O_{1s}	N_{1s}	S_{2p}
Binding energy (eV)	285.4	288.8	533.2	400.1	169.0
	± 0.3	±0.5	±0.3	± 0.1	± 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*	74.50	5.09	19.20	2.44	3.85

* 2 h modification time.

To further quantify the extent of the modification reaction, fluorohydroxybenzoic acids were used, 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 $g \cdot m^{-2}$ 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 ($\Delta L^* = 96.8 \pm 0.1$ and $\Delta E^* = 7.3 \pm 0.2$ for 4-fluoro-3-hydroxybenzoic acid and $\Delta L^* = 94.6 \pm 0.1$ and $\Delta E^* = 9.0 \pm 0.2$ for 3-fluoro-4-hydroxybenzoic acid).

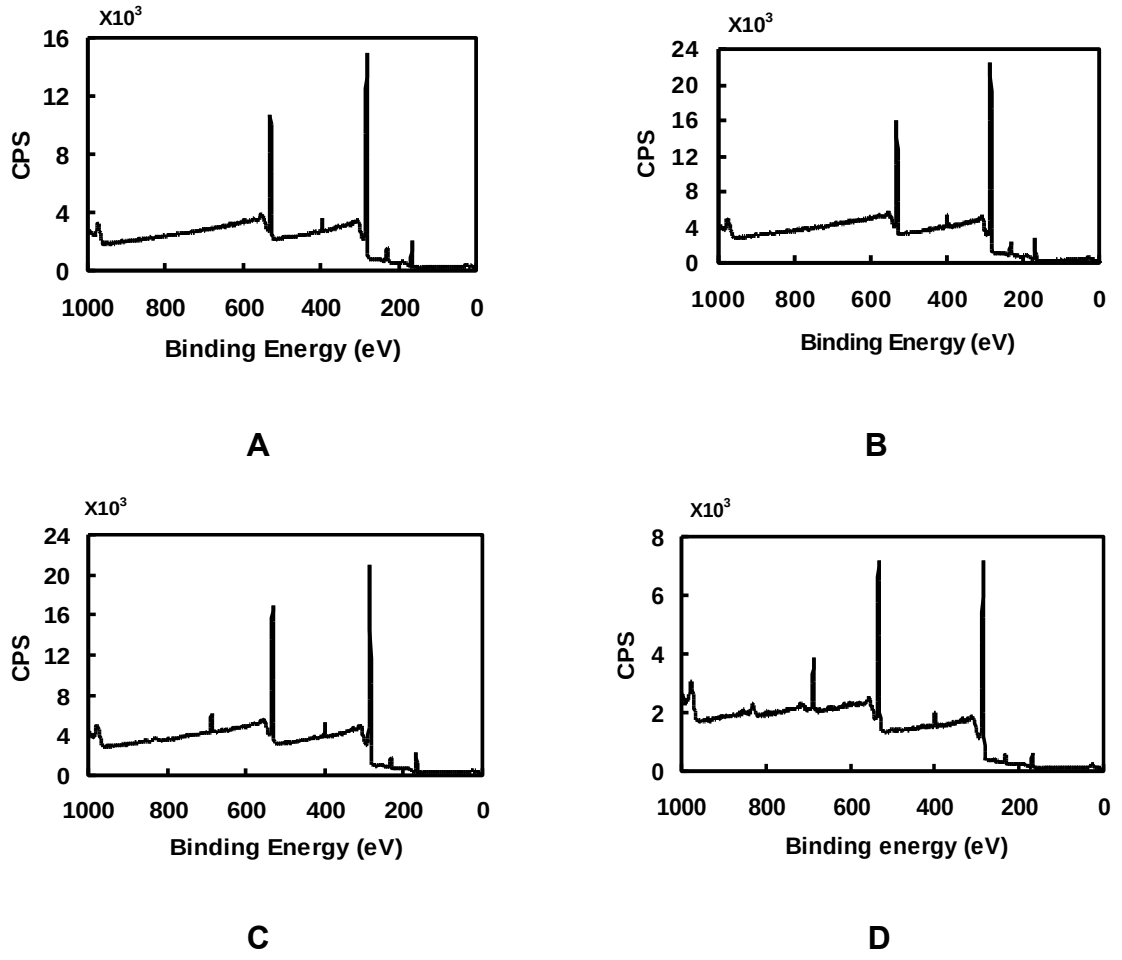


Figure 5. XPS spectra of unmodified and modified PES membranes with fluorinated hydroxybenzoic acids. Unmodified membrane (**A**); 2-fluoro-6-hydroxybenzoic acid (**B**); 4-fluoro-3-hydroxybenzoic acid (**C**); 3-fluoro-4-hydroxybenzoic acid (**D**).

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.

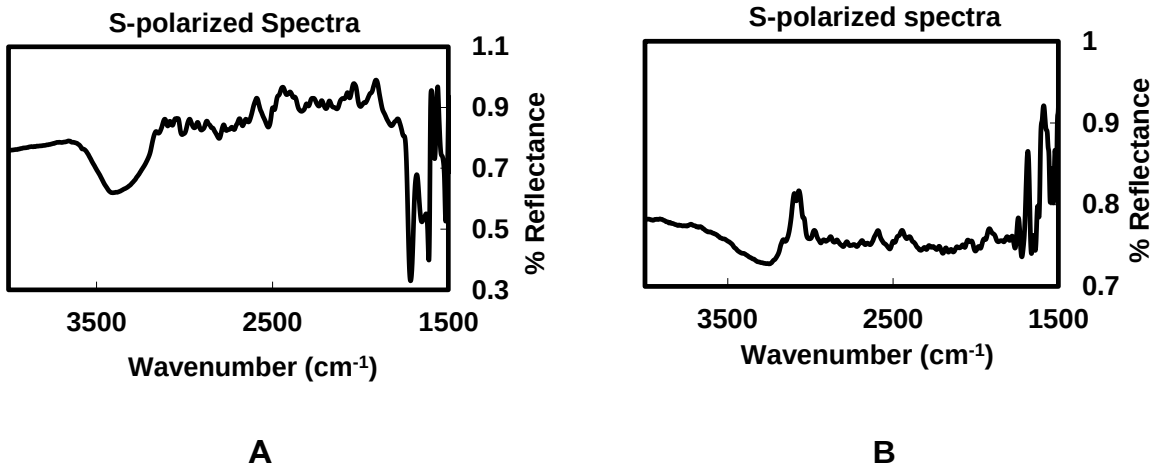


Figure 6. IRRAS spectra for 4-hydroxybenzoic acid (**A**) and gallic acid (**B**) modified membranes. Reflectance is relative to that of the unmodified membrane.

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).

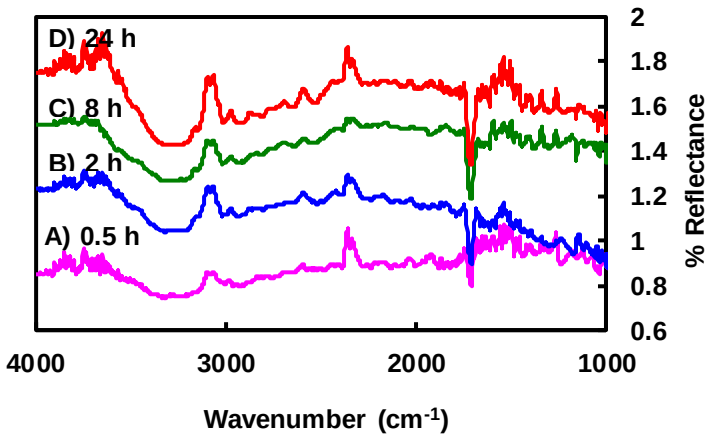


Figure 7. IRRAS spectra for 4-hydroxybenzoic acid-grafted membranes at 0.5 (**A**), 2 (**B**), 8 (**C**), and 24 h (**D**) modification time. Reflectance is relative to an unmodified membrane.

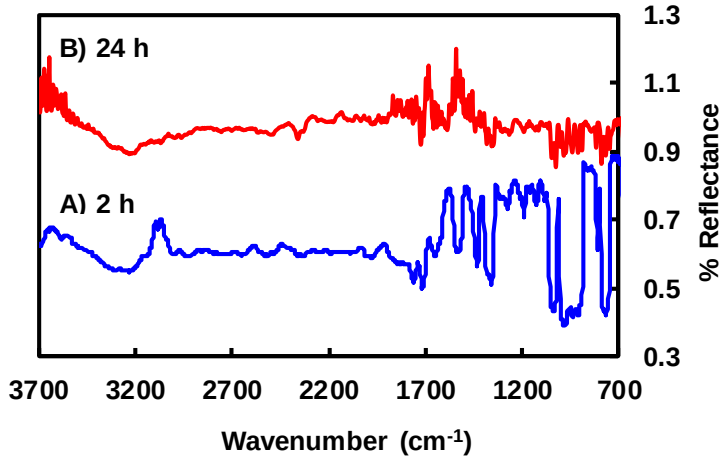
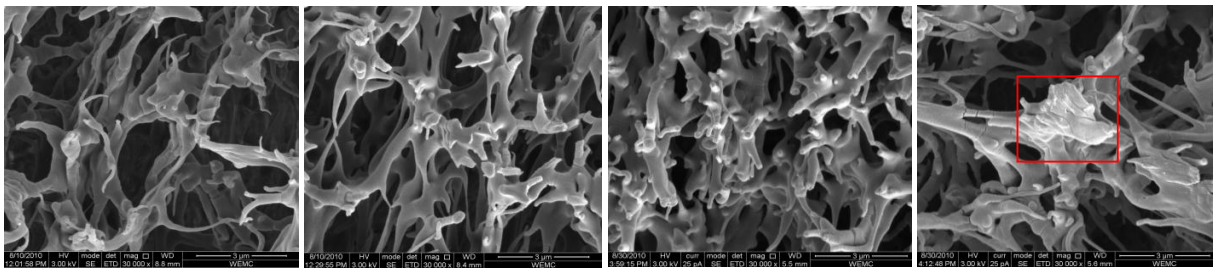


Figure 8. IRRAS spectra for gallic acid-grafted membranes at both 2 (**A**) and 24 h (**B**) modification time. Reflectance is relative to an unmodified membrane.

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 (Fig 9A-C). Only upon extended modification times for gallic acid (*e.g.*, 24 h; Fig 9D), larger structures that appear in an inhomogeneous fashion (red rectangle in Figure 9D) start to appear, pointing to the necessity to limit the modification time in this case to increase sample-to-sample reproducibility.



A B C D

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

3.1. 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.

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 towards 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.

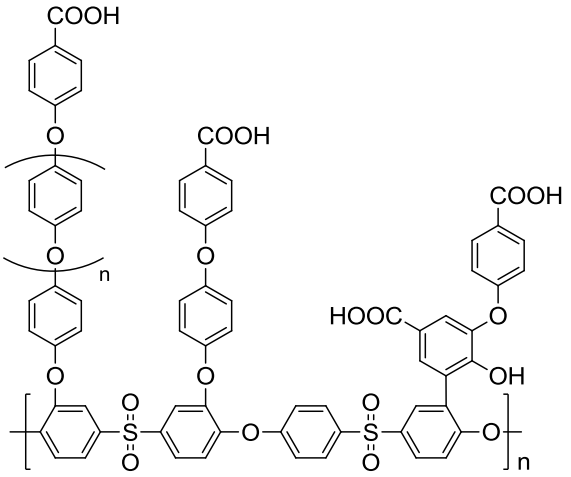


Figure 10. Schematic representation of a possible chemical structure of the PES surface after modification with 4-hydroxybenzoic acid, containing mainly O-linked structures with some C-linked compounds.

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} $mg\cdot nm^{-2}$ = 10 $mg\cdot m^{-2}$. The obtained coverage of PES membranes by *e.g.* hydroxybenzoic acid is in the range of hundreds of $mg\cdot m^{-2}$, and thus is on average significantly more than a monolayer.

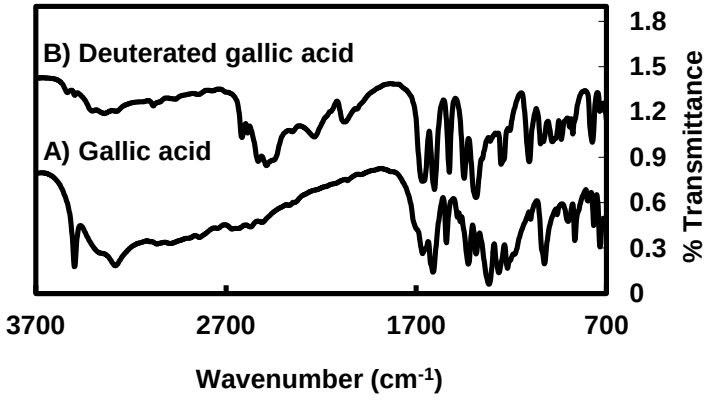


Figure 11. FT-IR spectra for gallic acid (**A**) and deuterated gallic acid (**B**), as KBr pellets.

3.2. 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 $mg \cdot m^{-2}$; 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 *e.g.* gallic acid mean that removal of the H on the -OH para to the COOH yields a radical that is 6.7 $kcal \cdot mol^{-1}$ more stable than the radical obtained by removal of the meta -OH hydrogen atom.

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

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 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 $kcal \cdot mol^{-1}$) 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.

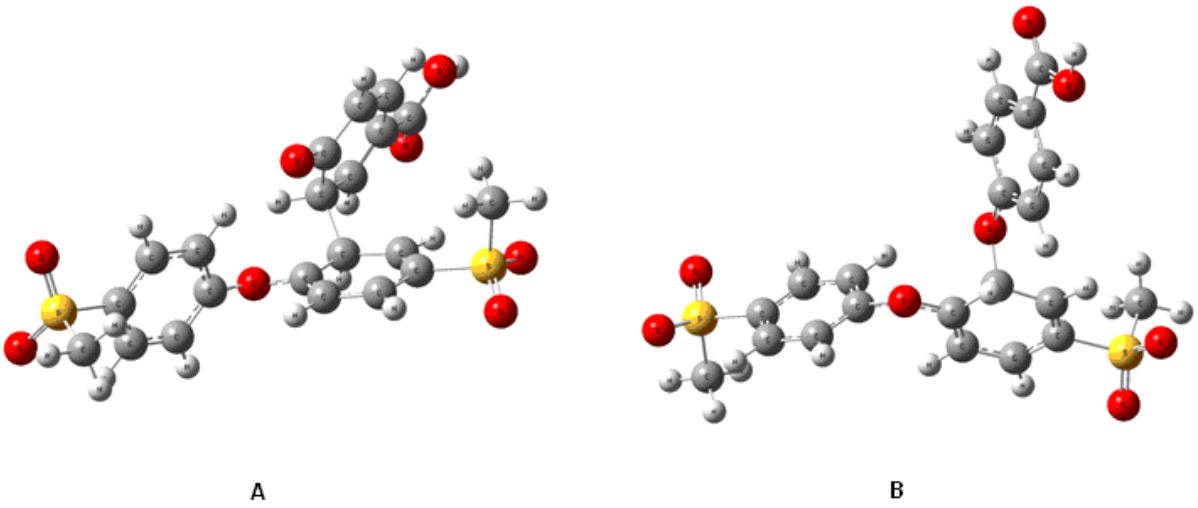


Figure 12. 4-Hydroxybenzoic acid coupled to PES polymer through (**A**) an aromatic carbon atom and (**B**) a phenolic oxygen atom.

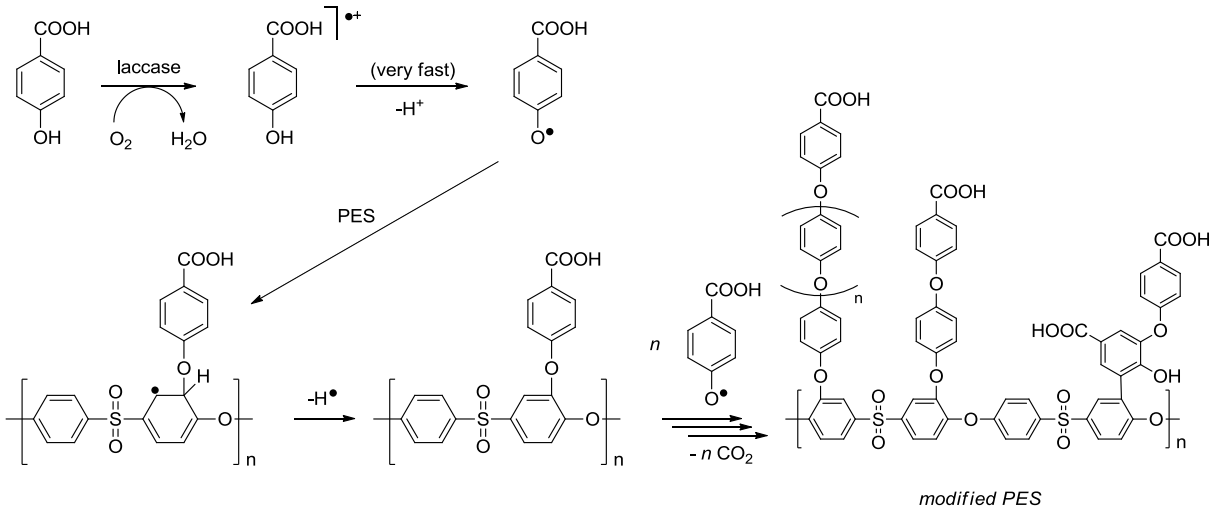


Figure 13. Tentative mechanism for the formation of reactive 4-hydroxybenzoic acid radicals by laccase and grafting of the radicals to PES membranes.

4. 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 firstly 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 pre-formed 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 $ml \cdot cm^{-2} \cdot min^{-1}$ 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 method for PES membranes can be tailored to serve various separation purposes.

5. 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.

Acknowledgments

The authors would like to thank ISPT for financial support of this research, and their project partners from Norit X-Flow and Friesland Campina for lively scientific discussions. We thank dr. Marcel Giesbers (XPS) and Tiny Franssen-Verheijen (SEM) for instrumental assistance.

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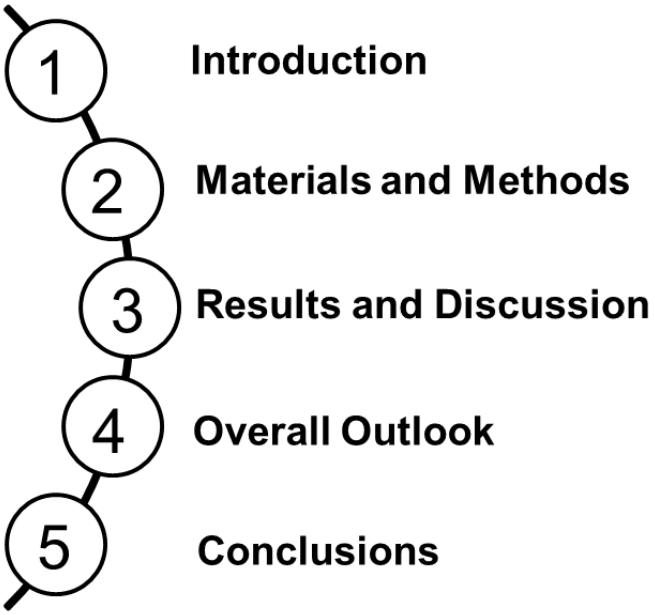
Chapter Four

Membrane Modification and Characterization



This chapter has been published as:

Laccase-Catalyzed Modification of PES Membranes with 4-Hydroxybenzoic Acid and Gallic Acid, Norhan Nady, Karin Schroën, Maurice Franssen, Mohamed S. Mohy Eldin, Han Zuilhof, and Remko Boom, J. Membr. Sci. 394-395 (2012) 69-79.

Laccase-Catalyzed Modification of PES Membranes with 4-Hydroxybenzoic Acid and Gallic Acid

Abstract

We here report on the performance of poly(ethersulfone) membranes modified with 4-hydroxybenzoic acid and gallic acid as substrates, and using laccase as biocatalyst under several modification conditions. The average flux of the base membrane was never reduced more than 20% (mostly below 10% reduction) by modification with 4-hydroxybenzoic acid, and not more than 9% for gallic acid. The mechanical and thermal properties of the membrane were not adversely affected by the modification method. For 4-hydroxybenzoic acid, longer modification times (*i.e.*, hours) and higher substrate concentrations lead to modified membranes with a better protein repellence. The reaction with gallic acid is faster, but less effective in terms of the resulting protein repellence.

In conclusion, the laccase-catalyzed modification of poly(ethersulfone) membranes is a mild method with low environmental impact that leads to effective protein repellence while keeping the bulk properties of the base membrane intact. This makes laccase-catalyzed modification an interesting alternative for currently used membrane modification methods.

1. Introduction

Membrane filtration is a key technology in many fields, such as food production, downstream processing in biotechnological processes, and water treatment [1-8]. A major obstacle is flux decline resulting from fouling, which necessitates frequent cleaning, and occasional replacement of the membrane, which represents the largest operating cost [9,10].

Protein adsorption is often the initial step in biofouling [11], and may result from different types of interactions with the membrane surface, such as Van der Waals interactions, electrostatic interactions and hydrogen bonding [12,13]. The frequently used polymer-based (*e.g.*, poly(arylsulfone) as investigated in this paper) membranes are appreciated for their high mechanical, thermal, and chemical stability, and their high flux-to-price performance. However, these materials are all more or less hydrophobic, which makes these membranes susceptible to fouling by proteins. In case of poly(ethersulfone) (PES), a blend with a hydrophilic polymer is used (*e.g.*, with polyvinylpyrrolidone, (PVP)), which renders the surface of the membrane much more hydrophilic, but which fundamentally changes the membrane structure as well [14]. In addition, use of PVP is not possible with every membrane-forming polymer, and even membranes with PVP often show extensive fouling by proteins [14,15]. Therefore, surface modification to influence and ideally prevent adsorption on the membrane surface is an attractive route [14], as long as the modification method does not influence the mechanical strength of the membrane.

Various investigations on surface grafting, *e.g.*, with gas plasmas and UV, have shown that an increased hydrophilicity of the membrane surface generally reduces membrane fouling [15-18]. On the other hand, steric hindrance (entropic repulsion of coated/grafted polymer chains) can drastically reduce protein adsorption, without influencing the overall hydrophilicity of the surface [19]. Thus, both surface structure and hydrophilicity have significant impact on membrane anti-fouling performance [20].

This thesis focuses on PES membranes. The incorporation of a wide variety of functional groups to these membranes, *e.g.*, through blending, coating, plasma and radiation induced-grafting, has been reported [21]. In general, these methods offer only limited control over the resulting surface structure, cannot be characterized as environmentally friendly due to the required use of harmful solvents and reactive or persistent chemicals, and they may negatively influence the mechanical properties of the membrane [14,21]. Enzyme-catalyzed, and in our case laccase-catalyzed modification makes use of naturally occurring, non-toxic reactants under mild reaction conditions in aqueous media [22].

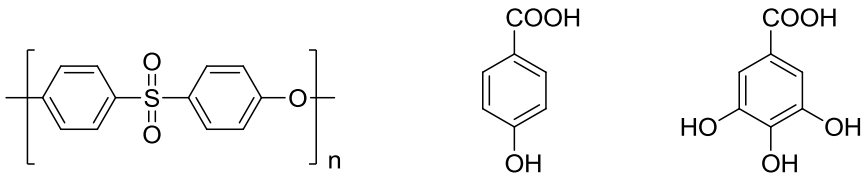
The enzyme laccase from the mushroom *Trametes versicolor* is able to oxidize phenolic compounds to yield reactive radicals [23-25]. These radicals can covalently bind to each other (polymerization), but we have found that they can also covalently bind to a PES membrane (grafting), mainly via their OH-groups [22]. In the current research we use 4-hydroxybenzoic acid and gallic acid as substrate for laccase, and evaluate modified PES membranes based on *e.g*. their flux and protein repellence. These substrates of laccase were chosen since their binding will add ionic and hydrogen bond-forming properties to the PES membranes.

We report here the effects of laccase-catalyzed modification of PES membranes at various modification times, substrate and enzyme concentrations, reaction temperatures, buffer strengths, and reaction pHs on membrane properties such as flux and protein repellence. In previous research [22], we found that PES membranes can be covalently modified with phenolic acids by the action of laccases. However, the flux, protein repellence, and (thermo)mechanical properties of the resulting membranes were not studied in detail yet. Therefore we here report on the performance of the modified membranes focusing on 4-hydroxybenzoic acid and gallic acid as modifiers. A variety of techniques was used to characterize the grafting yield, including quantified color changes and gravimetrically determined grafting yields. In addition, XRD, DSC, TGA, TMA, XPS, SEM and mechanical analyses were used to further characterize the modified membranes. Besides, the stability of the modification against strong base and acid was tested. All these aspects are brought together in an outlook section presented at the end of the results.

2. Materials and Methods

2.1. Chemicals

All chemicals were used as received. 4-hydroxybenzoic acid (99%): Alfa Aesar (The Netherlands), gallic acid (≥99.5%): Loba Chemie Pvt. Ltd (India), catechol (>98%): Oxford Laboratory Reagent (India), sodium acetate (anhydrous, ≥99%): Polskie Odczynniki Chemiczne S.A., (Poland), acetic acid (99.9%): Laboratory Chemicals (Egypt). Flat sheet commercial poly(ethersulfone) membrane: Sartorius (symmetric, 0.2 µm pore size, 50 mm diameter, 150 µm thickness, water flow rate > 28 $ml \cdot cm^{-2} \cdot min^{-1}$ at $\Delta P = 1$ bar). Laccase from *Trametes versicolor* (>21.4 $U \cdot mg^{-1}$, Fluka). Deionized water was used in all experiments. The chemical structures of the membrane material and the substrates are shown in Figure 1.



Poly(ethersulfone) (PES) 4-hydroxybenzoic acid gallic acid

Figure 1. Molecular structure of poly(ethersulfone), 4-hydroxybenzoic acid, and gallic acid.

2.2. Laccase Assay

The laccase activity was determined with catechol as substrate. The assay mixture contained 0.33 ml of 10 mM catechol, 2.67 ml of 0.1 M sodium acetate buffer (pH 5), with 0.025 $U \cdot ml^{-1}$ laccase. Oxidation of catechol is monitored by following the increase in absorbance at 400 nm ($\varepsilon = 26{,}000\ M^{-1} \cdot cm^{-1}$) [26], 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 catechol per min at 25 °C.

2.3. Membrane Modification

The flat sheet membranes were incubated in 40 ml sodium acetate buffer containing different concentrations of phenolic acid (4-hydroxybenzoic acid or gallic acid) and enzyme. Air was supplied as O_2 source and was used for gentle continuous mixing to ensure a homogenous reaction medium. The liquid was stirred for 1 min before the enzyme was added. After a specific modification time, the membranes were removed from the reaction medium and washed by 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. All used modification conditions are illustrated in the appendices; conditions written in bold italic font are repeated samples to investigate the reproducibility.

2.4. Color Measurements

The CIELAB coordinates for the modified membranes were measured with an X-Rite (SP62 Sphere Spectrophotometer, CIE L*a*b*, and ΔE* at D 65/10º). The color values L* (lightness), a* (red-green axes), b* (yellow-blue axes), and E* (the degree of total color change) were determined relative to the unmodified membrane as standard (compare mode; ΔL*, Δa*, etc). Aperture size was 8 mm diameter. Three readings were taken from three different places on each sample and the average value was calculated.

In order to remove any loosely bound material, the membranes were washed by filtration with at least 1000 ml deionized water before the actual color change was measured.

2.5. Pure Water Flux

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 ºC and 300 rpm.

The pure water flux is calculated with Equation 1, in which, J_w = water flux ($m^3 \cdot m^{-2} \cdot s^{-1}$), Q = quantity of permeate collected (m^3), Δt = sampling time (s), and A = the membrane area (m^2).

$$J_w = \frac{Q}{\Delta t \cdot A} \qquad \text{(Eqn. 1)}$$

2.6. 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 grafted membrane was washed by filtration of at least 1000 ml deionized water. Subsequently its weight was measured, after drying in a desiccator.

2.7. BSA Adsorption

BSA was used as a model compound to evaluate protein adsorption on unmodified and modified membranes. $1 g \cdot l^{-1}$ 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 followed by repeated (3×) immersion in deionized water and decantation.

The membrane flux after BSA adsorption was determined as follows. First, the membrane was backwashed using 100 ml of deionized water to remove any unbound BSA, and then forward washed 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. The reported values are the average of three independent measurements.

2.8. Scanning Electron Microscope (SEM)

Unmodified and modified membranes were imaged using a Scanning Electron Microscope (Jeol Jsm 6360LA, Japan). The membrane samples were cut using a very sharp shaving blade and were then coated with Au/Pt, and imaged at a voltage of 20/30 KV, and a resolution of 1280 × 960 pixels.

2.9. X-ray Photoelectron Spectroscopy (XPS) Analysis

A JEOL JPS-9200 X-ray Photoelectron Spectrometer (Japan) is used for 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 an analyzer pass energy of 10 eV. All high-resolution spectra were corrected with a linear background before fitting. The elemental composition analyses were done for a modified spin-coated PES layer on 1×1 cm^2 silicon dioxide slides. For this, 0.25 wt % PES in dichloromethane, was spun at 2500 rpm for 10 s, producing a PES layer with a thickness of around 20 nm. The PES coated strips were heated for 60 min at 300 °C, and then kept in glass-covered dishes within desiccators supplied with silica gel for drying. Three samples were modified independently for each condition.

The modified membranes (*i.e.*, not the spin-coated surfaces) were analyzed before and after washing with 0.2 M HCl or 0.2 M NaOH (pH 1 and 13), respectively. Each modified membrane was divided into three parts; two parts were washed with acid or base followed by immersion in the respective liquid for 15 min, the third part was kept as a reference. The modified PES membranes were kept in glass-covered dishes in desiccators containing silica gel.

2.10. Differential Scanning Calorimetery (DSC)

The glass transition temperature (T_g) was measured with differential scanning calorimetry (DSC, NETZSCH, DSC-200PC) at a heating rate of 10 °C·min^{-1}. The T_g was defined as the onset temperature of the change in heat capacity during the heating cycle.

2.11. Thermal Gravimetric Analysis (TGA)

TGA analysis of the blank and the modified membranes was carried out using a Thermal Gravimetric Analyzer (Shimadzu TGA-50, Japan). The samples were scanned over a temperature range from 20 to 700 °C at a temperature gradient of 10 °C·min^{-1} under nitrogen flow.

2.12. X-Ray Diffraction (XRD)

X-ray diffraction patterns for polymeric membranes were obtained on a Shimadzu XRD-7000 X-ray diffractometer using a CuKα radiation source operating at 40 kV and 30 mA. The samples were analyzed as such.

2.13. Thermomechanical Analysis (TMA)

The change in membrane thickness while subjected to increased load at a fixed temperature was measured with the Thermomechanical Analyzer (TMA-60H Shimadzu). Temperature range was 20-1500 °C and the force range was 0 ± 5N (± 500 gf).

2.14. Mechanical Properties

Samples were cut in a dog-bone-like shape. The total length of each sample was 37 mm, the gauge length of the samples was about 16 mm; the width was 13 mm at the top and 7.2 mm (narrowest) at the middle of the sample, to force a fracture in the middle of the sample. Tensile testing of the films was performed with the Texture Analyzer T2 (Stable Micro Systems, Ltd., Surrey, United Kingdom), at a constant crosshead speed of 0.1 mm·s^{-1} until breaking. Stress-strain curves were calculated from load-elongation curves measured for two samples from each film. The tensile strength, elongation at break, and Young's modulus were calculated from the stress-strain curves.

3. Results and Discussion

In this section we present the results obtained with 4-hydroxybenzoic acid and gallic acid as substrates (reactants) for laccase-catalyzed modification of PES membranes. We first discuss the substrates individually regarding flux, grafting yield, protein repellence, color change, and appearance. Next we compare the mechanical and thermal properties of the modified membranes, with culminates into an outlook for laccase-catalyzed modified membranes.

3.1. Membrane Modification with 4-Hydroxybenzoic Acid

PES membranes were modified with 4-hydroxybenzoic acid using laccase (see experimental section for details). Two reaction types compete during this treatment, namely the desired grafting reaction of monomer onto the PES membrane and the undesired reaction of the monomers to form homopolymer in solution [22]. The homopolymer may adhere to the surface, but will not be covalently bound to the modification layer. Therefore, the samples were analyzed both before and after extensive washing. The total color change (ΔE*) and grafting yield are illustrated in Figure 2 for two substrate concentrations as function of the modification time. The color change increases with increasing modification time and substrate concentration as shown in Figure 2A, and only a small amount of the modification layer is removed by washing. This indicates the formation of a grafted layer. As the modification time for the two 4-hydroxybenzoic acid concentrations increases, the washing step after modification removed a smaller fraction of the color: a longer modification time leads to a larger amount of strongly bound material. A higher substrate concentration leads to more color change, but also to a larger amount that can be washed off (see Figure 2B). Thus, at low concentration, less monomer binds, but it is mainly covalently bound, while at higher substrate concentration a larger fraction of substrate binds in the form of homopolymer, by physisorption. All the following data are determined after washing the membrane by a flux with 1000 ml deionized water.

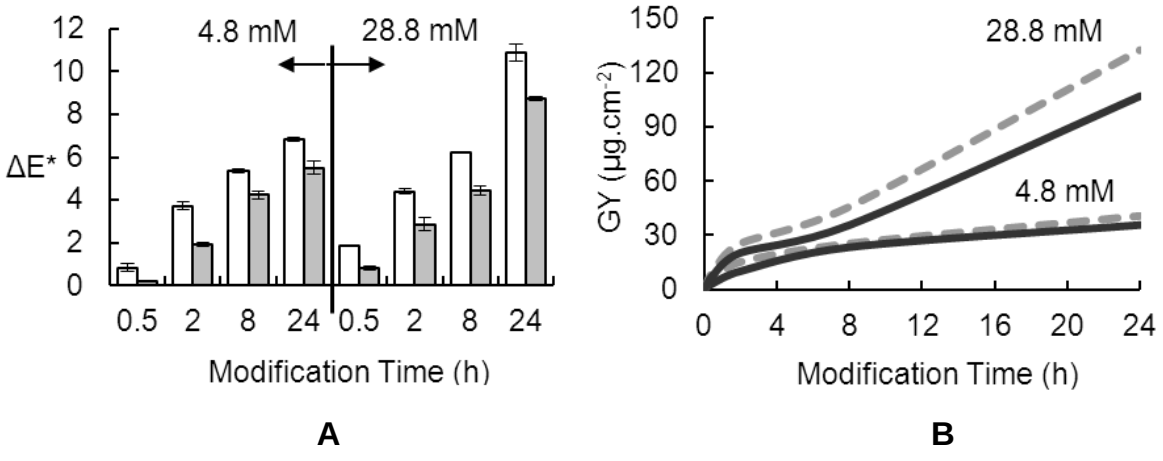


Figure 2. A: Total color change (ΔE*) before (white) and after washing (gray) of samples obtained with (4.8 and 28.8 mM) 4-hydroxybenzoic acid, after 0.5, 2, 8, and 24 h modification time. **B**: The grafting yield for the same samples, before (dashed line) and after (solid line) washing and filtration with water.

The gravimetric grafting yield was determined together with the color, the clean water flux, and the amount of BSA that adsorbs to the surface. The total change of color (ΔE*, Figure 3A) increased gradually with increasing grafting yield up to 30 $\mu g \cdot cm^{-2}$. At grafting yields that are much higher than those shown in Figure 3, the color increased more strongly with grafting yield, most probably due to formation of dense layers that are more saturated in color (*i.e.*, have higher extinction coefficients).

For all reaction conditions tested, the actual values obtained for color, grafting yield, flux and BSA adsorption are given in the Supporting Information (Appendices A and B). It is worth mentioning that the color developed more strongly at higher enzyme concentrations and temperatures (*i.e.* going from 25 to 55 °C), due to a faster overall reaction [27], but at temperatures above 55 °C, the enzyme is inactivated. The pH has a pronounced effect on the grafting yield: for example, at pH 4 the grafting yield after 2 h modification is 4.0 $\mu g \cdot cm^{-2}$, while at pH 5 it is 11.7 $\mu g \cdot cm^{-2}$ and 12.7 $\mu g \cdot cm^{-2}$ at pH 7 (while the fluxes are virtually the same as shown in Appendix A). This is attributed to the ionization of 4-hydroxybenzoic acid at higher pH, which leads to a lower oxidation potential, and therewith in a higher reaction rate and grafting yield [27-29]. The buffer strength [30] had minor influence on the grafting yield. The grafting yield did not influence the membrane flux to a great extent, as illustrated in Figure 3B. Typically the clean water flux reduction was below 10%; the highest clean water flux reduction at the highest grafting yield (107 $\mu g \cdot cm^{-2}$) was found to be 21.4% for 28.8 mM substrate in combination with 24 h modification (this point is not shown in Figure 3 but is included in Table 1). This implies that the original pores of the base membrane (0.2 μm) were only minimally constricted by the modification layer, in line with the formation of only relatively thin chemisorbed layers. The effect of a similarly sized modification layer will be bigger for small pores, and the resulting pore size distribution will be dominated more by the larger pores; however, given the thickness of the layer (<10 nm) and the average pore size (0.2 μm) the effect on pore size distribution is not expected to be very great.

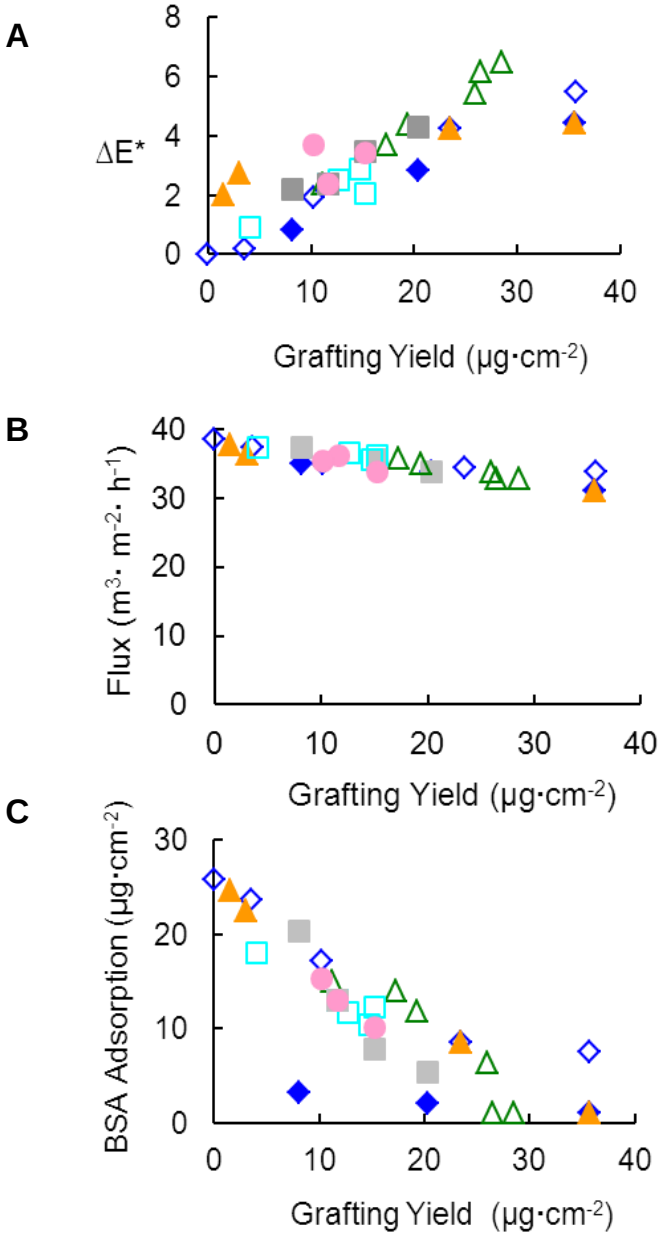


Figure 3. Total color change (ΔE*) (A), Clean water flux (B), and BSA adsorption (C) as a function of grafting yield; the reference modification condition is 4.8 mM 4-hydroxybenzoic acid, 0.5 U·ml^{-1} enzyme, 2 h modification time, 25 °C, pH 5, and 0.1 M sodium acetate buffer. The following parameters were varied: ◇ modification time (0.5, 2, 8 and 24 h) at 4.8 mM 4-hydroxybenzoic acid, ◆ modification time (0.5, 2, and 8 h) at 28.8 mM 4-hydroxybenzoic acid, ▲ 4-hydroxybenzoic acid concentration (0.6, 1.2, 4.8, and 28.8 mM) at 8 h modification time, ■ enzyme concentration (0.25, 0.75, and 1 U·ml^{-1}), △ reaction temperature (25, 35, 45, 55, and 65 °C), □ reaction pH (4-7), ● buffer strength (0.05, 0.1, and 0.5 M). Typical error: ± 0.2 for total color change (ΔE*), ± 0.3 m^3·m^{-2}·h^{-1} for flux, and ± 0.2 μg·cm^{-2} for BSA adsorption.

One could in fact give a sort of effective pore constriction, as the flux depends on the fourth power of the pore radius: assuming that all flux reduction is caused by narrowing of the pores due to deposition of the modification layer, the effective modification layer thickness can be estimated at 5 nm for 10% flux reduction to 12 nm for 21.4% flux reduction, for a membrane having 0.2 µm pores. Note that the values of the layer thicknesses used in this coarse estimate are in good agreement with those measured by ellipsometry (for further use in foulant studies carried out by reflectometry on non-porous flat PES surfaces), which is further discussed in chapter 5.

The adsorbed amount of bovine serum albumin (BSA, see Figure 3C) decreases with increasing grafting yield, and is close to zero after 8 h modification time at 28.8 mM 4-hydroxybenzoic acid. Similar effects were also observed in the previously mentioned follow-up reflectometry study in which we used non-porous surfaces; the observed effects are clearly due to surface modification, and not (so much) due to a reduction of hydrodynamic forces.

The blank membranes that did not receive any modification are prone to flux reduction (24%) due to BSA adsorption; modified membranes always showed higher residual fluxes as shown in Table 1. If the total flux loss (flux loss due to modification plus flux loss due to BSA adsorption) is compared to the original flux, most modified membranes give higher residual fluxes than the blank membrane, in spite of the (small) inherent flux reduction by the modification itself.

SEM images of membranes modified with 4.8 mM 4-hydroxybenzoic acid were used to investigate the change in morphology of the membranes as a result of the modification. As shown in Figure 4 (A - E), the thickness of the lamellae between the pores seems to increase with increasing modification time. With longer modification time (2 h), individual domains with clear edges can be seen, most probably consisting of homopolymer that was formed in solution and adsorbed to the membrane. At 8 and 24 h modification, these domains become more pronounced. On membranes modified with 28.8 mM 4-hydroxybenzoic acid for 24 h, film formation was noted, most probably as a combination of surface modification and homopolymer deposition (SEM image not shown). This latter membrane has the strongest measured flux reduction (21.4%). Low reaction pH (4) results in the formation of a very thin layer over the membrane surface (see image F in Figure 4), while increasing the pH from 5 to 7 seems to result in binding of small lumps of material (images G and H), we interpret to be most likely homopolymer.

Table 1. Flux reduction (% relative to blank) due to modification and protein adsorption.

	4.8 mM 4-hydroxybenzoic acid			28.8 mM 4-hydroxybenzoic acid		
Modification time (h)	Flux reduction due to modification [a]	Flux reduction due to BSA adsorption [b]	Total flux reduction [c]	Flux reduction due to modification [a]	Flux reduction due to BSA adsorption [b]	Total flux reduction [c]
0	0.0	23.8	23.8	0.0	23.8	23.8
0.5	3.4	6.3	9.5	9.5	3.0	12.2
2	9.5	8.6	17.2	12.2	2.9	14.8
8	10.8	4.4	14.8	19.4	2.7	21.6
24	12.2	0.0	12.2	21.4	3.0	23.8

Typical errors: [a] ± 0.1, [b] ± 0.3, and [c] ± 0.4

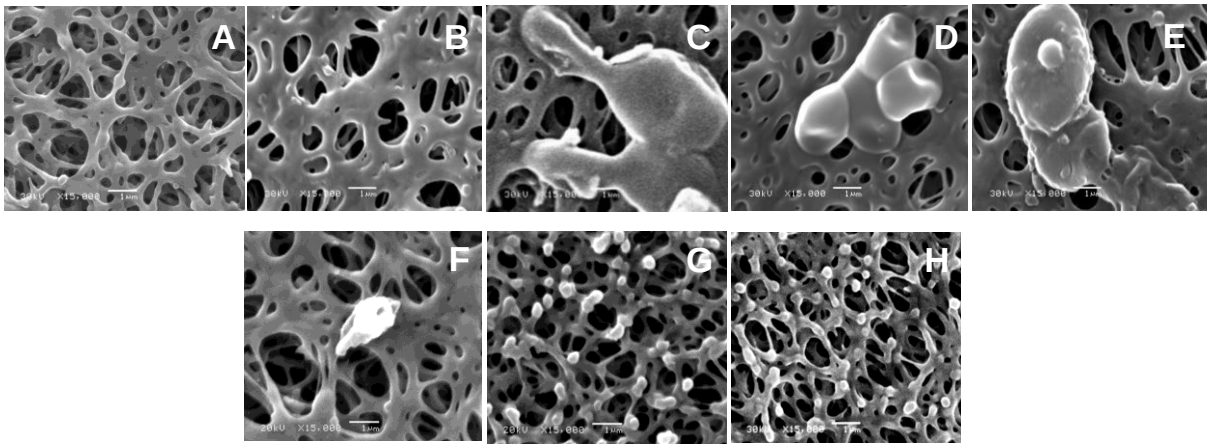


Figure 4. SEM images (15000× magnification, scale bar is 1 µm) for blank membrane (**A**), and 4-hydroxybenzoic acid modified membranes. The reference modification condition is 4.8 mM substrate, 0.5 U·ml^{-1} enzyme, 2 h modification time, 25 °C, pH 5, and 0.1 M sodium acetate buffer. Images **B**-**E** show the effect of different modification times (0.5, 2, 8, and 24 h), images **F**, **G**, and **H** show the modification at different pH values (4, 6, and 7, respectively).

3.2. Membrane Modification with Gallic Acid

Gallic acid has a slightly lower ionization potential than 4-hydroxybenzoic acid due to the two additional hydroxyl groups (see Figure 1), which leads to faster reaction. Therefore shorter modification times of up to 120 min were used to minimize the physisorption of homopolymers, which were dominant after 24 h modification time with gallic acid [22]. Similar effects were found as with 4-hydroxybenzoic acid: Figure 5A shows that ΔE* increases gradually with grafting yield. The concentration of gallic acid has an influence on the grafting yield, which increased from around 30 to 100 µg·cm^{-2} when increasing the gallic

acid concentration from 4.8 to 28.8 mM (actual experimental values are shown in appendix B).

In spite of the high grafting yield, the membrane flux was hardly influenced. The maximum clean water flux loss is 9% (effective layer thickness 5 nm) and the clean water flux even slightly *increased* in case of using a high concentration of gallic acid (28.8 mM) as shown in Figure 5B. This surprising result may be attributed to a better wetting of the pores by an increase in the number of OH groups inside the membrane pores (internal surface), opening up a larger fraction of all pores for permeation. A similar effect has been seen by Mathias Ulbricht and co-workers [31] when using high concentrations of three-armed cross linker with photo-initiated grafted PEGMA, which did not occur when using a two-armed cross linker. This suggests a change in the polymer structure.

The flux reduction due to protein adsorption of membranes modified with gallic acid was always lower than with unmodified membranes (Table 2). However, it did not reduce BSA adsorption to the same extent as 4-hydroxybenzoic acid, as shown in Figure 5C.

This can be understood as follows: phenolic acid radicals generated by laccase mainly react via their hydroxyl groups [22]. Gallic acid has three adjacent hydroxyl groups on its aromatic ring and as a result the formed radical can be further oxidized to an *o*-quinone [32]. This oxidation can occur inside the active site of the enzyme, or in solution by a disproportionation reaction between two radicals. Though they are less reactive than radicals, *o*-quinones still readily react via addition reactions in a Michael-type fashion [33] (see Figure 6).

If this happens to gallic acid (derivatives) on the membrane, extensive cross-linking may occur leading to rather compact layers (see Figure 7 for illustration of the effect). This is also supported by the XPS analyses of blank and modified spin-coated PES layers on silicon dioxide slides, and ellipsometry analysis (for experimental details see [34]; actual XPS graphs not shown here), which allows measurement of the elemental composition of the layer; results are summarized in Figure 7. In case of gallic acid, the sulfur signal is shielded by the modification layer, even though the layer is rather thin. The grafting yield increases with increasing modification time from 7 min to 2 h; however, the thickness of the layer (Th.) is reduced from 5.5 to 3.3 ± 0.2 nm, which may be attributed to collapse of the poly(gallic acid) layer due to cross-linking, and possibly to adsorption of polymers containing *o*-quinones. In spite of these differences, the sulfur signal is very comparable for both samples.

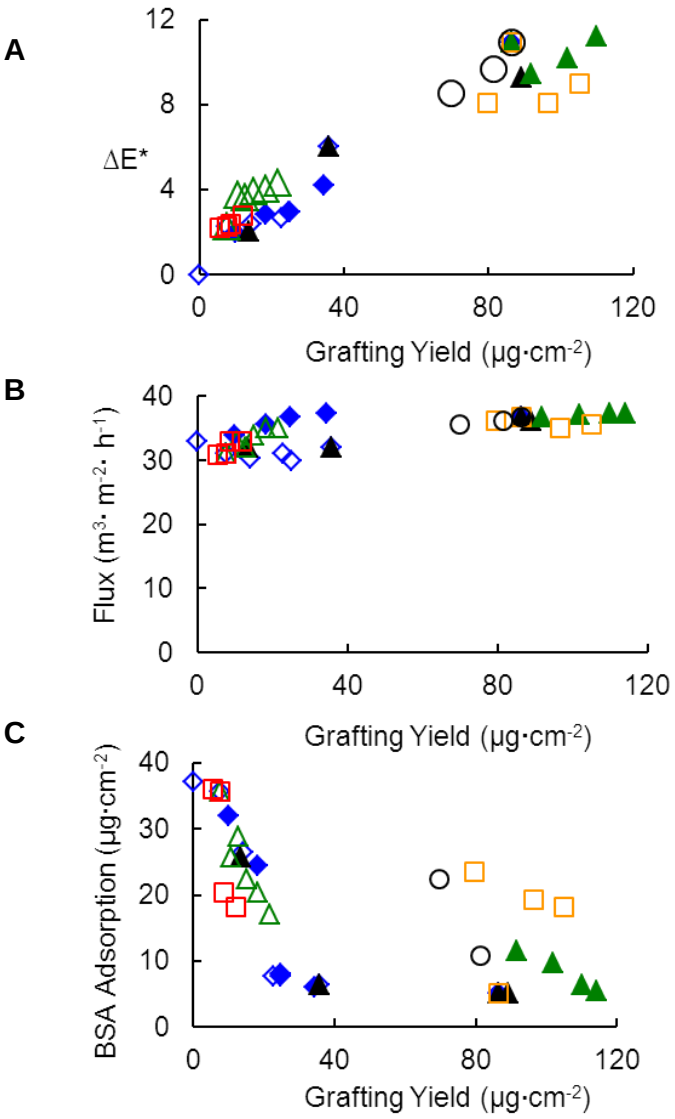


Figure 5. Total color change (ΔE*) (A), Clean water flux (B), and BSA adsorption (C) as a function of grafting yield; the reference modification condition is 0.5 U·ml^{-1} enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer with 4.8 and 28.8 mM gallic acid. The following parameters were varied: modification time (10, 20, 30, 60 and 120 min) with ◇ 4.8 mM gallic acid, and ◆ with 28.8 mM gallic acid, ▲ gallic acid concentration (1.2, 4.8, and 28.8 mM) at 120 min modification time, □ enzyme concentration (0.25, 0.5, 0.75, and 1 U·ml^{-1}) at 4.8 mM gallic acid and 10 min modification time, reaction temperature (25, 35, 45, 55, and 65 °C) at both △ 4.8 mM gallic acid and 10 min modification time and ▲ 28.8 mM gallic acid and 120 min modification time, □ reaction pH (4, 5, 6, and 7) at 28.8 mM gallic acid and 120 min modification time, ○ buffer strength (0.05, 0.1, and 0.5 M) at 28.8 mM gallic acid and 120 min modification time. Typical errors: ± 0.2 for total color change (ΔE*), ± 0.1 m^3·m^{-2}·h^{-1} for flux, and ± 0.3 μg·cm^{-2} for BSA adsorption.

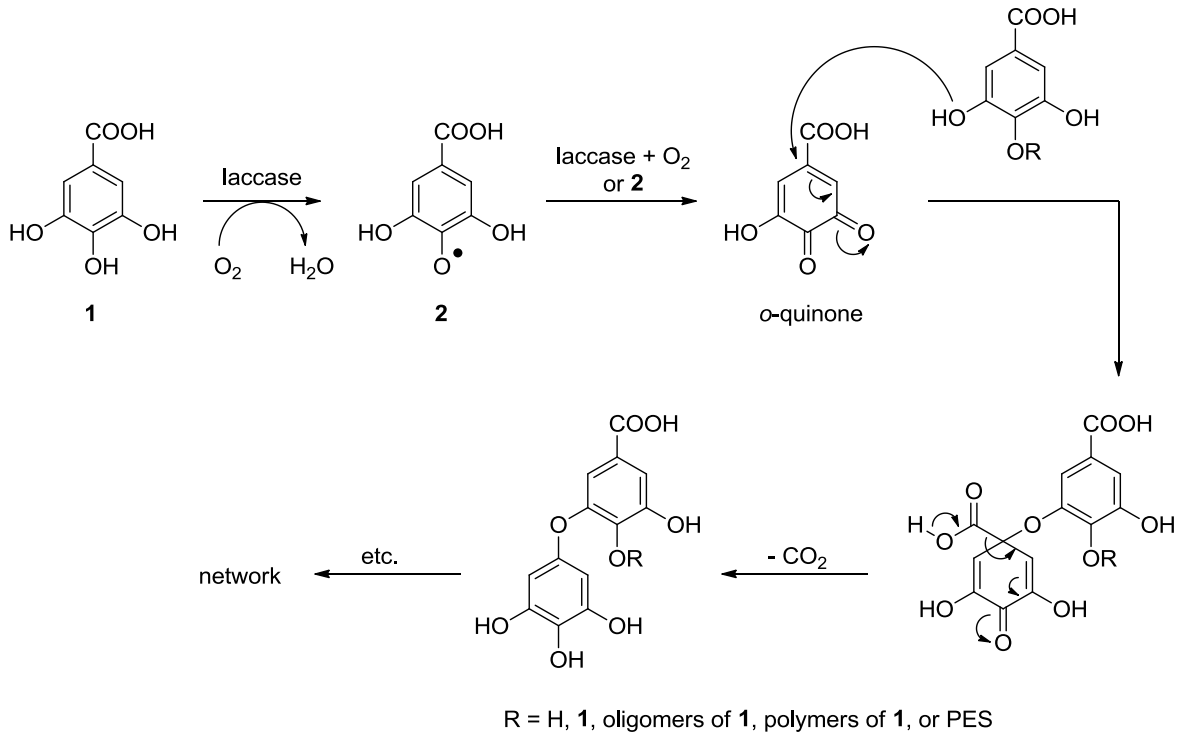


Figure 6. Proposed mechanism of the laccase-mediated formation of an *o*-quinone from gallic acid, and its reaction with gallic acid (derivatives) in solution or with the PES membrane.

Table 2. Flux reduction (% compared to blank) due to modification and protein adsorption. *Increases in flux are indicated by #.*

	4.8 mM gallic acid			28.8 mM gallic acid		
Modification time (min)	Flux reduction due to modification [a]	Flux reduction due to BSA adsorption [b]	Total flux reduction [c]	Flux change due to modification [a]	Flux reduction due to BSA adsorption [b]	Total flux change [c]
0	0.0	24.5	24.5	0.0	24.5	24.5
10	5.6	18.2	22.8	3.0#	8.3	5.6
20	8.1	9.8	17.1	7.9#	4.5	3.0#
30	9.4	3.6	12.8	11.4#	1.5	9.7#
60	5.6	2.7	8.1	13.3#	1.6	11.5#
120	2.9	1.4	4.3	11.4#	0.3	11.1#

Typical errors: [a] ± 0.1, [b] ± 0.1, and [c] ± 0.4

In case of 4-hydroxybenzoic acid, the increase in grafting yield is accompanied by an increase in the layer thickness from 4.7 ± 0.4 to 5.8 ± 0.2 nm and reduction in the detected percentage of sulfur (S_{2p}) from 3.4 ± 0.2 to 2.4 ± 0.3. 4-Hydroxybenzoic acid has only one hydroxyl group, so cross-linking via oxygen atoms is not possible. Cross-linking via carbon atoms is still possible but is less likely because the spin density in the 4-hydroxybenzoic acid radicals in highest at oxygen [chapter 3]. Branching however is still possible (see Figure 7) as well as collapse of longer chains, all leading to an increase in density.

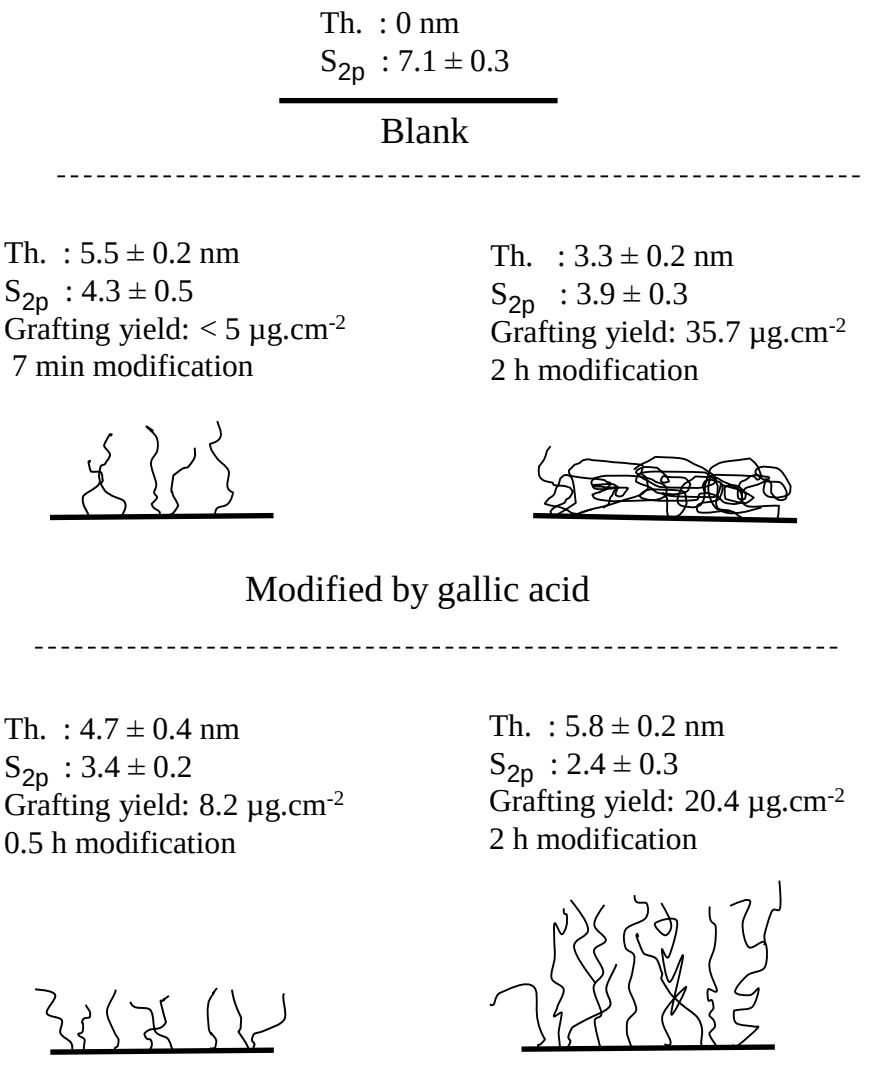


Figure 7. Schematic representation of the effect of modification conditions on the density (compactness) of the modification layer as deduced from atomic % of sulfur as determined by XPS (S_{2p}), layer thickness (Th.) determined by ellipsometry, and grafting yield (from real membranes) in case of PES layers modified with 4-hydroxybenzoic acid (28.8 mM) and gallic acid (4.8 mM).

The layer itself has a relatively loose structure, as the polymer itself has a good interaction with water, and will function to some degree as an entropic brush. Onto a compact layer as formed with gallic acid, this steric repulsion between extended chains and proteins is less effective, and the reduction of adsorption is mainly due to an increase in hydrophilicity. Thus, BSA will adsorb better on layers made from gallic acid. For that reason, a low grafting yield may be preferred with gallic acid, because it will keep more open structure with more hydroxyl groups on the surface, and possibly reduced BSA adsorption. For 4-hydroxybenzoic acid, the amount of grafted material seems to determine the length and density of the entropic brush and therewith its efficiency in protein repellence.

SEM images of the grafted PES membranes do not show the big lumps that were observed with 4-hydroxybenzoic acid at any of the chosen conditions (see Figure 8). The reaction of gallic acid is very fast and the modification process seems to be initiated across the entire membrane surface at the same time. Some tiny lumps appeared at higher temperatures (images I and J) and under other conditions at which homopolymer is most likely to be formed (higher substrate concentrations with long modification time, see images E and F), but in general no lumps were observed, which is in contrast to the SEM images in Figure 4 for 4-hydroxybenzoic acid.

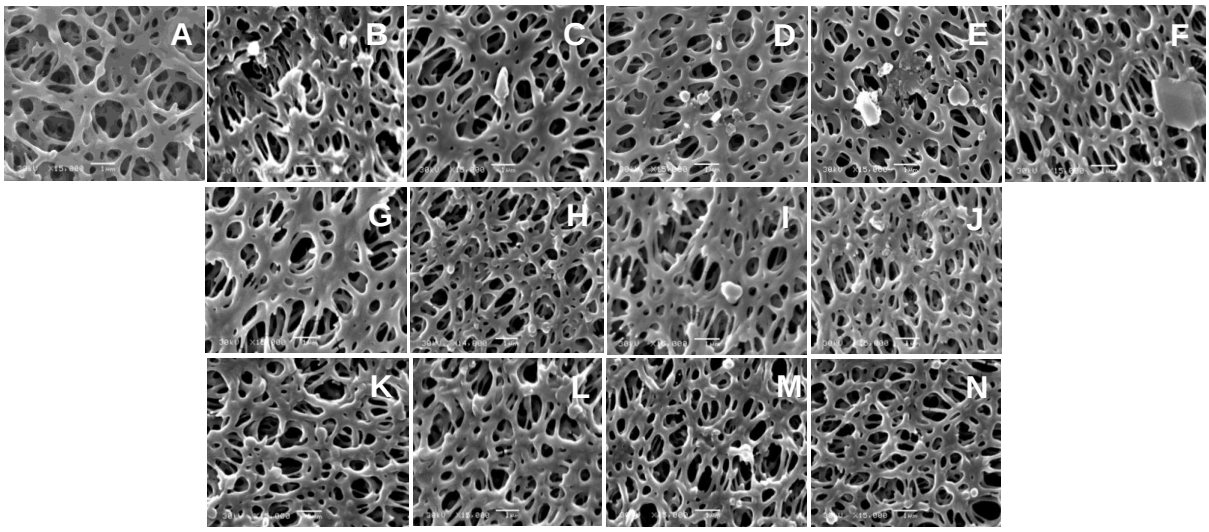


Figure 8. SEM images (15000× magnification, scale bar is 1 µm) for the unmodified membrane (image **A)**, and the gallic acid modified membranes. The reference modification condition is 0.5 $U \cdot ml^{-1}$ enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer. Images **B**-**F** show the effect of different modification times (10, 20, 30, 60 and 120 min) with 28.8 mM gallic acid. The effect of modification temperature (35, 45, 55, 65 °C) with 4.8 mM gallic acid and 10 min modification time is given in images **G**-**J**, and with 28.8 mM gallic acid and the same temperatures at 120 min modification time in images **K**-**N**.

3.3. Membrane 'Bulk' Properties

Blank PES membranes have a characteristic peak at 18.6° in XRD analysis [35]. The characteristic peaks for both phenolic acids are 17.6°, 24.5°, and 30° (see Figure 9) [36]. The peak around 17° corresponds to the benzene ring, and is present in the spectra of the membrane and the phenolic acids. The other two peaks are specific for the phenolic acids. The intensity of these peaks increases with the amount grafted onto the membrane. For 4-hydroxybenzoic acid the peaks were relatively small compared to the base PES polymer (about 1% grafted material; lines b and c), whereas for gallic acid (lines d and e), due to its fast reaction, this increased to about 2.8%.

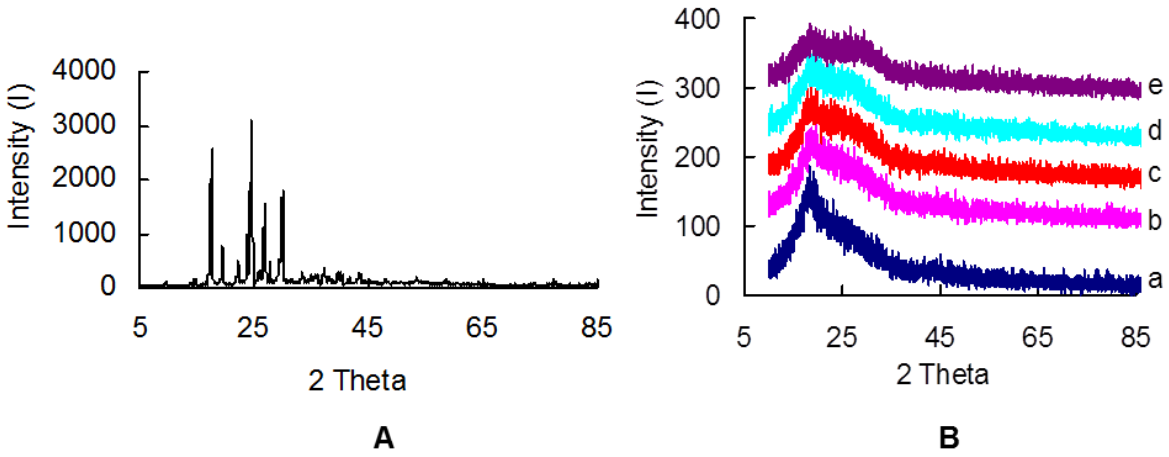


Figure 9. XRD analysis of 4-hydroxybenzoic acid (**A**) and modified membranes (**B**); blank PES membrane (**a**), membranes modified with 4.8 and 28.8 mM 4-hydroxybenzoic acid (**b** and **c**), membranes modified with 4.8 and 28.8 mM gallic acid (**d** and **e**). Modification conditions: 2 h modification time, 0.5 U·ml^{-1} enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer.

The results for TGA analysis are shown in Figure 10. For blank PES membranes [35], the effect seen between 400-600 °C corresponds to sulfur dioxide cleavage and ether bond cleavage. At even higher temperatures, the backbone (benzene ring) decomposes. When comparing with the blank membrane, the temperature at which the first significant weight loss occurs is increased by about 50 °C upon modification. This shows that the stability of the membrane is at least not negatively affected by the modification layer.

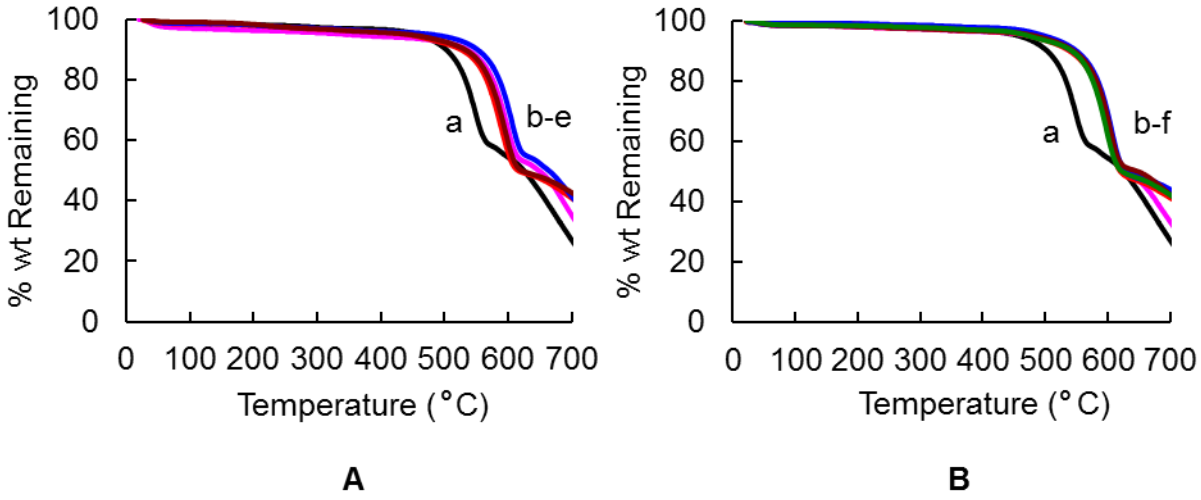


Figure 10. TGA analysis of PES membrane. Blank: (**a**) in both **A** and **B**. Graph **A**: membranes modified with 4-hydroxybenzoic acid; 28.8 mM at 0.5, 2, 8, and 24 h, respectively (**b**-**e**). Graph **B**: membranes modified with gallic acid; 4.8 mM at 10, 20, and 60 min modification time, respectively (**b**, **c**, and **d**), 28.8 mM at 10 and 30 min modification time, respectively (**e** and **f**). Modification conditions: 0.5 $U \cdot ml^{-1}$ enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer.

DSC analysis revealed that the glass transition temperature (T_g: onset temperature) for the blank PES membrane was 228 °C [37], and decreases only very slightly upon modification (Figure 11; 225-222 °C for 4-hydroxybenzoic acid-modified membranes, and 228-223 °C for gallic acid-modified membranes, respectively).

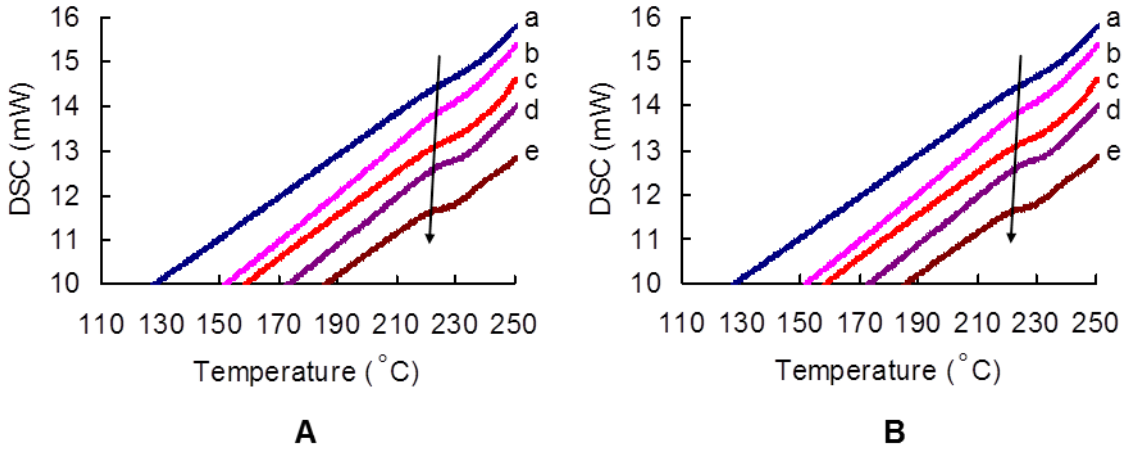


Figure 11. DSC analysis of PES membrane. Blank: (**a**) in both **A** and **B**. Graph **A**: membranes modified with 4-hydroxybenzoic acid, 4.8 mM (**b** & **d**) and 28.8 mM (**c** & **e**) at 0.5 and 2 h modification times, respectively. Graph **B**: membranes modified with 4.8 mM gallic acid at 10, 20, 30, and 60 min modification time, respectively (**b**-**e**). Modification conditions: 0.5 $U \cdot ml^{-1}$ enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer.

In addition, TMA analysis of the PES base membrane leads to noticeable reduction in the membrane thickness especially with the first 50 gram force (primary compaction) as shown by curve a in Figure 12. TMA analysis of the modified membranes proved that the PES base membranes were not negatively affected by the modification. In fact, they even became slightly stronger, and can resist a primary compaction at increasing load (see Figure 12 b-e as examples), as was also reflected in the ultimate strength and elastic modulus (data not shown). This all indicates that the grafting process does not affect the bulk properties of the membrane significantly.

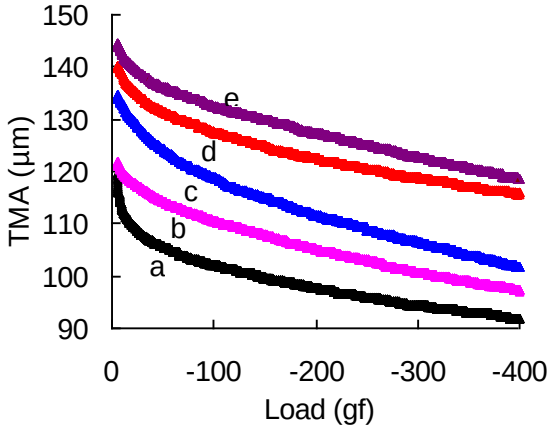


Figure 12. TMA analysis of blank PES membrane (**a**), membranes modified for 0.5, 2, 8, and 24 h with 4.8 mM 4-hydroxybenzoic acid (**b-e**), at 0.5 U·ml^{-1} enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer.

For a few modified membranes, the stability against treatment with strong acid (pH 1) and strong base (pH 13) was investigated using XPS (Table 3). Both the relative amounts of carbon and oxygen are markedly changed because of the added modification layer. The change in the atomic % after washing by acid or base relative to unwashed membrane may be attributed to (partial) degradation of the modification layer (and/or the base membrane). The modification layer appeared to be generally resistant towards strong acid but somewhat vulnerable towards strong base, depending on the modification conditions. These results are promising for cleaning conditions used in industrial practice.

Table 3. Analysis of XPS of blank and modified PES membranes before and after washing using 0.2 M HCl [pH 1] and 0.2 M NaOH [pH 13]. Modification conditions: 0.5 $U \cdot ml^{-1}$ enzyme, at 25 °C, pH 5, and 0.1 M sodium acetate buffer

Membrane. no.		Binding energy (ev)	C1s 285.4 ± 0.5 -C-C-	O1s 531 ± 0.3 -C-O-
	Sample		**Atomic %**	
1	*Blank*		74.3 ± 0.1	18.3 ± 0.04
	After acid wash		73.7 ± 0.2	18.3 ± 0.01
	After base wash		74.5 ± 0.1	17.8 ± 0.1
	Modified membranes			
2	28.8 mM 4-HBA + 2 h modification		68.6 ± 0.7	23.5 ± 0.4
	After acid wash		69.8 ± 0.6	22.7 ± 0.5
	After base wash		71.2 ± 0.1	20.8 ± 0.1
3	4.8 mM gallic acid+ 7 min modification		68.3 ± 0.3	22.7 ± 0.6
	After acid wash		68.4 ± 0.4	22.8 ± 0.6
	After base wash		69.1 ± 0.3	22.6 ± 0.4
4	4.8 mM gallic acid+ 20 min modification		73.9 ± 0.5	18.6 ± 0.1
	After acid wash		74.1 ± 0.5	18.4 ± 0.5
	After base wash		74.8 ± 0.5	17.6 ± 0.02

± xx is the variance between the two sides of the membrane
The rest of the 100% atomic analysis is for N1s and S2p.

4. Overall Outlook

The modification method shown in this chapter is a nice example of green chemistry, which uses non-toxic natural materials to replace environmentally detrimental routes (a comparison of modification methods can be found in [14]). The laccase enzyme uses naturally occurring phenols and oxygen from air to produce covalently modified poly(ethersulfone) membranes, with water as the only by-product. The typical times used for the process in this paper are below 2 hours, which makes the method interesting for practical application, but the required time could be reduced further if more enzyme were to be used. Obviously, optimization of grafting yield and structure would be needed as demonstrated in this paper.

The difference in the number of hydroxyl groups per molecule between 4-hydroxybenzoic acid and gallic acid (see Figure 1) affects the structure and shape of the modification layer and consequently the surface behavior against adsorption of BSA. A schematic overview of the reaction modes of the two substrates is given in Figure 13. In case of 4-hydroxybenzoic acid,

the presence of only one hydroxyl group leads to growth of the chains mostly in one dimension (possibly with branches), whereas the three hydroxyl groups of gallic acid likely induce growth in three dimensions and network formation. Besides, gallic acid is known to lead to abundant homopolymer formation in solution, which leads to more and stronger physisorption to the surface than 4-hydroxybenzoic acid [22]. Although homopolymer can be removed by rinsing, it does reduce the efficiency of the modification, and is considered an undesirable effect.

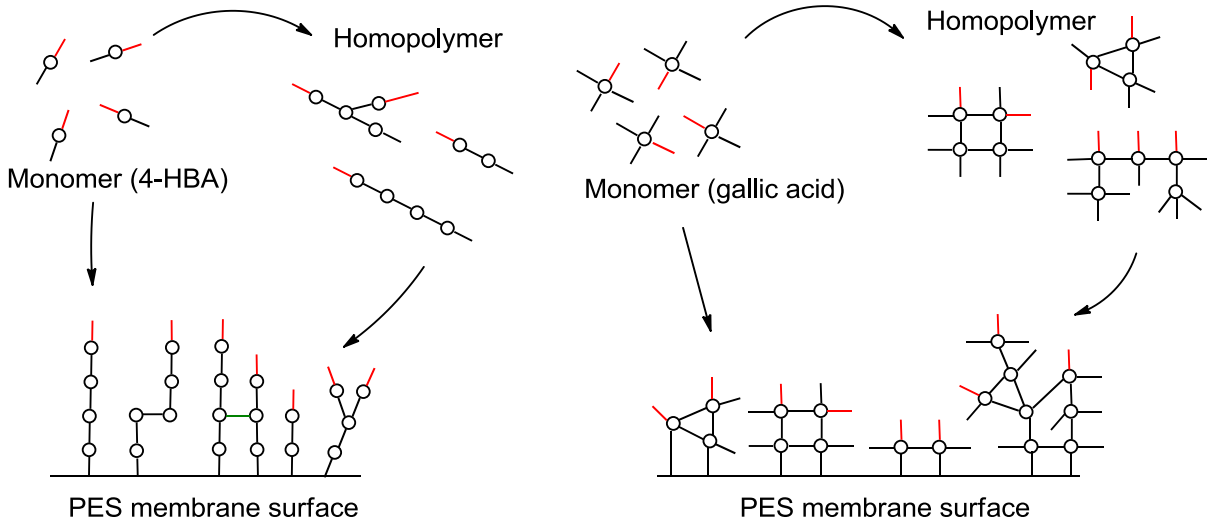


Figure 13. Schematic representation of modification layers formed by 4-hydroxybenzoic acid (4-HBA) and gallic acid. Black line: OH group or ether linkage; green line: C-C bond; red line: COOH group.

The results show that one can influence the layer structure by changing the reactivity of the reactants, for example by changing the number of reactive groups per molecule. Substrates with a large number of reactive groups will lead to a denser 3D network (in polymer terminology, a thermosetting material), while molecules with only one reactive group can only give linear structures with some branches, which may swell and extend in water and give rise to entropic repulsion (entropic brush formation). These findings are not only important in membrane separation, but can be used to modify any poly(ethersulfone) surface and tune its surface properties at will.

5. Conclusions

The results presented in this paper show that PES membranes can be successfully modified with phenolic components using the enzyme laccase and environmentally benign reactants

under very mild conditions. The modified membranes showed a considerably reduced protein adsorption (and in some cases even no detectable protein adsorption at all), while neither the clean water flux nor the bulk properties were significantly influenced.

The nature of the substrate molecule can be used to control the morphology and functionality of the modified PES surface. Relatively open, swollen layers are obtained with 4-hydroxybenzoic acid; denser layers are obtained with gallic acid. Even though the latter membrane shows less reduction in protein adsorption, the clean water flux remains stable and high. The flexibility of this very mild modification method opens a broad area of interesting applications for poly(ethersulfone) membranes and other processes in which poly(arylsulfone) is used.

Acknowledgments

The authors thank the team (Mahmoud Darwesh, Nahla Taha and Nagla Roushdy) of the central labs in the Advanced Technology and New Materials Research Institute (ATNMRI), Alexandria, Egypt for performing SEM, DSC, TGA, TMA and XRD analyses, and dr. Marcel Giesbers (WUR) for performing XPS analyses. Finally, we thank ISPT for financial support of this research.

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Appendix A. Overview of modification conditions used for Figure 3. ***Bold italic data: repeated control samples (a, b, and c).*** Substrate: 4-hydroxybenzoic acid (4-HBA), modification conditions were 4.8 mM substrate concentration, 2 hours modification time, 0.5 U·ml^{-1} enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer, unless stated otherwise.

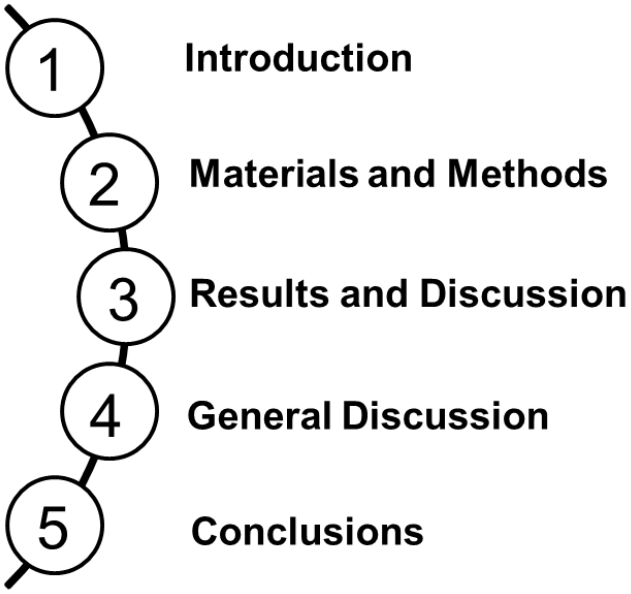
Symbol	Modification time (h)	4-HBA concentration (mM)	Enzyme concentration (U·ml^{-1})	Modification temperature (°C)	pH	Buffer strength (mM)	Grafting yield (µg·cm^{-2})	ΔE* Total color change	Flux (m^3·m^{-2}·h^{-1})	BSA adsorption (µg·cm^{-2})
◇	0	4.8	0.5	25	5	0.1	0.0	0.0	38.6	25.7
	0.5	.	.	.	.	.	3.6	0.2	37.3	23.6
	2	.	.	.	.	.	10.2[a]	1.9	35.0	17.2
	8	.	.	.	.	.	23.4[b]	4.2	34.5	8.6
	24	.	.	.	.	.	35.7[c]	5.5	33.9	7.5
◆	0	28.8	0.5	25	5	0.1	0.0	0.0	38.6	25.7
	0.5	.	.	.	.	.	8.1	0.8	35.0	3.2
	2	.	.	.	.	.	20.4	2.9	33.9	2.1
	8	.	.	.	.	.	35.7	4.5	31.1	1.1
	24	.	.	.	.	.	107.0	8.7	30.4	Undetectable
▲	8	0.6	0.5	25	5	0.1	1.5	2.0	37.8	24.7
	.	1.2	.	.	.	.	3.0	2.7	36.5	22.5
	.	***4.8***	***.***	***.***	***.***	***.***	***23.3[b]***	***4.2***	***34.6***	***8.6***
	.	***28.8***	***.***	***.***	***.***	***.***	***35.5[c]***	***4.5***	***31.1***	***1.1***
■	2	4.8	0.25	*25*	5	0.1	8.1	2.2	37.3	20.4
	.	.	0.75	.	.	.	15.3	3.5	35.6	7.9
		.	1.00	.	.	.	20.4	4.3	33.9	5.4
△	2	4.8	0.5	30	5	0.1	17.3	3.7	35.8	13.9
	.	.	.	35	.	.	19.4	4.4	35.0	11.8
	.	.	.	45	.	.	26.0	5.4	33.9	6.4
	.	.	.	55	.	.	28.5	6.5	32.9	1.1
	.	.	.	65	.	.	26.5	6.2	32.9	1.1
□	2	4.8	0.5	25	4	0.1	4.0	0.9	37.3	18.1
	.	***.***	***.***	***.***	***5***	***.***	***11.7[a]***	***2.9***	***35.6***	***13.0***
	.	.	.	.	6	.	14.8	2.5	36.7	10.4
	.	.	.	.	7	.	12.7	2.1	36.1	11.7
●	2	4.8	0.5	25	5	0.05	15.3	3.4	33.9	10.2
	.	***.***	***.***	***.***	***.***	***0.1***	***11.7[a]***	***2.4***	***35.6***	***13.0***
	.	.	.	.	.	0.5	10.2	3.7	35.4	15.3

Appendix B. Overview of modification conditions used for Figure 5. ***Bold italic data: repeated control samples (a, b, c, and d).*** Substrate: Gallic acid, modification conditions were 0.5 U·ml^{-1} enzyme, 25 °C, pH 5, and 0.1 M sodium acetate buffer, unless stated otherwise.

Symbol	Modification time (min)	Gallic acid concentration (mM)	Enzyme concentration (U·ml^{-1})	Modification temperature (°C)	pH	Buffer strength (mM)	Grafting yield (µg·cm^{-2})	ΔE* Total color change	Flux (m^3·m^{-2}·h^{-1})	BSA adsorption (µg·cm^{-2})
◇	0	4.8	0.5	25	5	0.1	0.0	0.0	32.9	37.2
	10	.	.	.	.	.	7.6[a]	2.3	31.1	35.7
	20	.	.	.	.	.	14.3	2.4	30.3	26.5
	30	.	.	.	.	.	24.9	3.0	29.9	8.1
	60	.	.	.	.	.	22.4	2.7	31.1	7.6
	120	.	.	.	.	.	35.7[b]	6.0	32.0	6.5
◆	0	28.8	0.5	25	5	0.1	*0.0*	*0.0*	*32.9*	*37.2*
	10	.	.	.	.	.	10.0	2.0	33.9	32.1
	20	.	.	.	.	.	18.3	2.8	35.5	24.4
	30	.	.	.	.	.	24.8	3.0	36.7	7.6
	60	.	.	.	.	.	34.4	4.2	37.3	6.1
	120	.	.	.	.	.	86.6[c]	10.9	36.7	5.0
▲	120	1.2	0.5	25	5	0.1	13.6	2.0	32.0	25.7
	.	***4.8***	***.***	***.***	***.***	***.***	***35.6[b]***	***6.0***	***32.0***	***6.5***
	.	***28.8***	***.***	***.***	***.***	***.***	***86.6[c]***	***10.9***	***36.7***	***5.0***
□	10	4.8	0.25	*25*	5	0.1	5.5	2.2	30.8	36.0
	.	***.***	***0.50***	***.***	***.***	***.***	***7.6[a]***	***2.3***	***31.1***	***35.7***
	.	.	0.75	.	.	.	8.7	2.4	32.9	20.4
	.	.	1.00	.	.	.	11.9	2.8	32.9	18.2
△	***10***	***4.8***	***0.5***	***25***	***5***	***0.1***	***7.5[a]***	***2.3***	***31.1***	***35.7***
	.	.	.	30	.	.	12.7	3.6	32.0	28.9
	.	.	.	35	.	.	10.7	3.7	32.9	25.8
	.	.	.	45	.	.	15.2	3.9	33.9	22.5
	.	.	.	55	.	.	18.4	4.1	34.9	20.4
	.	.	.	65	.	.	21.6	4.3	35.0	17.2
▲	***120***	***28.8***	***0.5***	***25***	***5***	***0.1***	***86.6[c]***	***10.9***	***36.7***	***5.0***
	.	.	.	35	.	.	91.8	9.4	36.7	11.6
	.	.	.	45	.	.	102.0	10.2	37.0	9.6
	.	.	.	55	.	.	110.0	11.2	37.3	6.4
	.	.	.	65	.	.	114.3	12.2	37.3	5.4
□	120	28.8	0.5	25	4	0.1	79.7	8.1	36.1	23.6
	.	***.***	***.***	***.***	***5***	***.***	***86.6[c]***	***10.9***	***36.7***	***5.0***
	.	.	.	.	6	.	96.6	8.1	35.0	19.3
	.	.	.	.	7	.	105.2	9.0	35.5	18.3
○	120	28.8	0.5	25	5	0.05	69.8	8.6	35.5	22.5
	.	***.***	***.***	***.***	***.***	***0.1***	***86.6[c]***	***10.9***	***36.7***	***5.0***
	.	.	.	.	.	0.5	81.5	9.7	36.1	10.7

Chapter Five

Adsorption on Modified Model PES Surfaces



This chapter will be submitted as:

Enzyme-Catalyzed Modification of PES Surfaces: Reduction in Adsorption of BSA, Dextrin and Tannin, Norhan Nady, Karin Schroën, Maurice Franssen, Remco Fokkink, Mohamed Mohy Eldin, Han Zuilhof, and Remko Boom.

Enzyme-Catalyzed Modification of PES Surfaces: Reduction in Adsorption of BSA, Dextrin and Tannin

Abstract

Poly(ethersulfone) (PES) can be modified in a flexible manner using mild, environmentally benign components such as 4-hydroxybenzoic acid and gallic acid, which can be attached to the surface via catalysis by the enzyme laccase. This leads to grafting of mostly linear polymeric chains (for 4-hydroxybenzoic acid, and for gallic acid at low concentration and short modification time) and of networks (for gallic acid at high concentration and long exposure time). The reaction is stopped at a specific time, and the modified surfaces are tested for adsorption of BSA, dextrin and tannin using *in-situ* reflectometry and AFM imaging.

At short modification times, the adsorption of BSA, dextrin and tannin is significantly reduced. However, at longer modification times, the adsorption increases again for both substrates. As the contact angle on modified surfaces at short modification times is reduced (indicative of more hydrophilic surfaces), and keeps the same low values at longer modification times, hydrophilicity is not the only determining factor for the measured differences. At longer modification times, intra-layer reactivity will increase the amount of cross-linking (especially for gallic acid), branching (for 4-hydroxybenzoic acid) and/or collapse of the polymer chains. This leads to more compact layers, which leads to increased protein adsorption.

The modifications were shown to have clear potential for reduction of fouling by proteins, polysaccharides, and polyphenols, which could be related to the surface morphology.

1. Introduction

Membrane fouling is a serious problem in membrane filtration. The first step of membrane fouling is adsorption of components from the feed. This adsorption process depends on the nature of the components, the (surface) material of the membrane, and on the operating conditions [1-5]. Poly(ethersulfone) (PES) is a popular material for membranes, as it lends itself well for membrane preparation through phase inversion, and yields mechanically and chemically robust membranes. However, its hydrophobicity makes it (as usually proposed) intrinsically susceptible to adsorption by *e.g.* proteins [4,6,7]. Besides proteins, also other foulants such as polysaccharides and polyphenols pose problems in practice, and have been investigated individually [8-10] or in combination [11,12].

Obtaining a (more) hydrophilic membrane surface has been the aim of many researchers in order to reduce (or even prevent) adsorption [13-15], but the precise mechanism of fouling by adsorption is complex. Hydrogen bonding, hydrophobic interactions, π-π stacking and changes in water structure at the membrane have been proposed as (parts of) adsorptive mechanisms [9,10,16]. In addition, a decrease in the Gibbs energy (G) of the system (*i.e.*, protein, surface, and solvent) leads to adsorption of foulants. For that, any change in the enthalpy, entropy, and the system temperature should affect the adsorption process [17,18]. For example, the release of water from the surface or protein molecules (*i.e.*, dehydration processes) with a concomitant large entropy gain leads to increased protein adsorption and an overall decreased Gibbs energy [17-19]. On the other hand, the surface *structure* is also sometimes considered as a factor to influence protein adsorption [5,17,20-22]. Steric hindrance and the osmotic effect of hydrated coated/grafted polymer branches on the surface contributes to the reduction of adsorption by keeping the foulant molecules at a distance behind a barrier of adsorbed water molecules (*i.e.*, hydration layer). Moreover, the strength and thickness of this hydration layer has been used to effectively repel proteins from surfaces covered with zwitterionic polymer brushes [23]. In addition to these parameters, the adsorption process can also be affected by the protein structure, protein stability, concentration, pH, and ionic strength [3,17,19].

While proteins may initially adsorb due to hydrophilic interactions, their irreversible adsorption is typically caused by hydrophobic interactions. For polysaccharides, hydrophobic interactions with the membrane surface are important, while hydrogen bonding is primarily responsible for the adsorption of polyphenols on hydrophilic membrane surfaces [16,24]. To

cover a wide range of fouling interactions, this study thus includes a selection of model foulants from each category.

The enzymatic modification of PES membranes allows the formation of hydrophilic layers onto the membrane surface from monomers (4-hydroxybenzoic acid, gallic acid, and other phenolic compounds) using oxidative enzymes such as laccase [25]. This very mild procedure (room temperature, neutral pH) only involves natural chemicals and the enzyme. The resulting surface is rather polar, and its low contact angle (52-62º) suggested the potential use as antifouling surface [25,26]. However, the exact influence of the surface modification on the adsorption of fouling constituents has not yet been evaluated.

In this work we aim to quantify these antifouling effects with reflectometry and ellipsometry. We use a PES layer on top of a silicon dioxide surface as a model PES surface for characterization of the adsorption of various components. These PES model surfaces were then further modified with the enzyme laccase and phenolic acids as described previously [25,26]. Reflectometry is a widely used technique to investigate the initial adsorption of solutes [16,27-31] onto silicon dioxide surfaces: the technique allows the *in-situ* measurement of the dynamics and total quantity of the (ir)reversible adsorption of foulant monolayers. The adsorption of BSA, dextrin, and tannin was used as model materials for proteins, polysaccharides, and polyphenols, respectively. Ellipsometry was used to measure the thickness and refractive index of each layer (silicon dioxide, PES, and modification layer) on the silicon substrate. Contact angle measurements were used to characterize the hydrophilicity of the surface, and AFM to evaluate the surface structure in detail. This complete set of data allows a general delineation of the potential of modified PES layers as antifouling surfaces.

2. Materials and Methods

2.1. Chemicals

Poly(ethersulfone) (PES) (Ultrason, E6020P) was obtained from BASF (Ludwigshafen, Germany). Prime grade 150 mm silicon wafers of type P/B <100> orientation, thickness 660-690 µm, with a 2.5 nm native oxide layer were purchased from Wafer Net Inc (San Jose, CA, USA). From Sigma-Aldrich were purchased: catechol (>99%), sodium acetate (anhydrous, ≥99%), sodium phosphate dibasic anhydrous (>99%), acetic acid (99.9%), 4-hydroxybenzoic acid (99%), dichloromethane ACS (stabilized, 99.9%), gallic acid (>97.5%), sodium phosphate monobasic (>98%), Bovine Serum Albumin, Cohan Analog (>98%), and laccase from *Trametes versicolor* (>20 $U \cdot mg^{-1}$). Tannic acid powder (>98%) was obtained from

Riedel-de Haën (Seelze, Germany), and dextrin 5 (from maize starch, >95%) was purchased from Fluka (Neu-Ulm, Germany). All chemicals were used as received. Milli-Q water was used throughout.

2.2. Laccase Assay

The laccase activity was determined with catechol as substrate. The assay mixture contained 0.33 ml of 10 mM catechol, 2.67 ml of 0.1 M sodium acetate buffer (pH 5), with 0.025 $U \cdot ml^{-1}$ laccase. Oxidation of catechol is monitored from the absorbance at 400 nm (ε = 26,000 $M^{-1} \cdot cm^{-1}$) [32] after 20 min. One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 µmol of catechol per min at 25 °C.

2.3. Preparation of Model Surfaces and Layer Thickness

The thickness of the native silicon dioxide layer (2.5 nm) was increased to approximately 70 ± 2 nm (triplicate measurements on ≥ 3 positions per strip) by heating at 1000 °C for 100 min. Then, the wafers were cut into strips of 1 × 4.5 cm. The strips were sonicated in ethanol for 15 min, washed with water and ethanol, and dried in flowing nitrogen. The thickness and refractive index of silicon dioxide layer were determined with a computer-controlled null ellipsometer (Sentech instruments Gmbh) at λ = 632.8 nm and angle of incidence 70°. Values of 3.85 and 0.02 were used for the refractive index (*n*) and the imaginary refractive index (*k*) of silicon [27,28], respectively. Subsequently the strips were given a plasma treatment (PDC-32G, Harrick at RF-level high) for 10 min. After the removal of any dust by using a flow of nitrogen, the strips were used as substrate for the model PES surfaces by spin coating them with 0.25% w/w PES solution in dichloromethane for 10 s at 2500 rpm. The spin coating was performed immediately after plasma cleaning. After spin coating, the PES coated strips were put at 300 °C for 60 min.

The thickness and refractive index of PES layers deposited on silicon dioxide were determined by ellipsometry. To ensure homogenous PES layers of around 20 nm, the thickness was tested at 3 different locations; the variation was never more than 2 nm. The spin-coated PES model surfaces were then modified using laccase (see respective section). The surfaces were kept for 24 h in glass-covered dishes in desiccators supplied with silica gel to remove any moisture. After drying, the thickness and refractive index of the modification layer was determined by ellipsometry, as the average of values measured at three different locations. The refractive index initial values used for the silicon dioxide, PES, and modification layers were 1.46, 1.65, and 1.57, respectively [27,33].

2.4. Modification Experiments

The spin-coated PES model surfaces were incubated in 20 ml 0.1 M sodium acetate buffer containing different concentrations of phenolic acids (substrates or monomers) and laccase enzyme. A flow of air was used for mixing and as oxygen source. After a specific modification time, the modified model surfaces were removed from the liquid and washed by strong flushing with Milli-Q water. Gallic acid and 4-hydroxybenzoic acid were used as enzyme substrates, at two concentrations (4.8 and 28 mM), together with various modification times ranging from a few minutes to 24 h [25,26]. The modified PES model surfaces were kept in glass-covered dishes in desiccators supplied with silica gel for drying.

2.5. Static Water Contact Angle

Blank and modified PES model surfaces were characterized by static water contact-angle measurement using a Krüss DSA 100 apparatus. Drops of demineralized water (7 µL) were deposited on three different spots of each surface, and the contact angle was calculated from three different measurements taken on each of two independently modified surfaces.

2.6. Atomic Force Microscopy (AFM)

Spin coated PES model surfaces were prepared on strips (1 × 6 cm) that were half covered with polymer solution. In this way, blank and modified surfaces are present on the same strip, and images of both were obtained with an MFP-3D AFM from Asylum Research (Santa Barbara, CA). Imaging was performed in AC mode in air using OMCL-AC240 silicon cantilevers (Olympus Corp.). In order to investigate the effect of the modification layer on protein adsorption, the half-modified strips (surfaces) were incubated in 50 ml of 0.1 $g \cdot l^{-1}$ BSA solution for 1 h followed by immersion and flushing with Milli-Q water, and stored as described before. The root mean squares (RMS) profiles (*i.e.*, roughness) of the two parts were calculated before and after exposure to BSA solution.

2.7. Adsorption Measurements

In Figure 1A, the reflectometry set-up is schematically depicted. A monochromatic light beam (1) (He-Ne laser; 632.8 nm) is linearly polarized and passes a 45° glass prism (2). This beam arrives at the interface (3) with an angle of incidence close to 71° for the solvent/substrate interface. After reflection at the interface and refraction at the prism, the beam is split into its parallel and perpendicular components (p- and s-polarized) of which the intensities are measured continuously (I_p and I_s) relative to the plane of incidence by means of

a beam splitter (4). Both components are detected separately by two photodiodes (5) and the ratio between the intensity of the parallel and perpendicular components is recorded. The output signal S is the ratio between the two intensities (I_p/I_s) (6). The surface is inside a stagnation point flow-cell allowing homogeneous adsorption on surfaces. Two 4-way valves (7) are used to switch between the sample solutions.

Solutions of BSA (0.1 $g \cdot l^{-1}$), dextrin, and tannin (both 0.2 $g \cdot l^{-1}$) were prepared in 10 mM phosphate buffer pH 7. Before the start of the adsorption experiments, each slide (model surface) was incubated for 45 min in buffer to avoid artefacts due to initial surface wetting. All solutions were degassed using ultrasound. After placing the slide inside the flow cell, the buffer solution was injected until the output signal was nearly constant. This value was taken as the base signal, S_0. Each adsorption experiment was conducted in three stages: 15 min buffer injection (base-line) followed by 15 min foulant solution injection (adsorption process; the maximum adsorbed amount was mostly reached within 15 min) and then 15 min buffer re-injection (desorption process). The change in intensity of the reflected polarized laser that followed was converted into adsorbed mass using a 5-layer matrix model [27,28]. This model uses equation (1), where Γ is the adsorbed amount ($mg \cdot m^{-2}$), Q_f is a sensitivity factor ($mg \cdot m^{-2}$), and S_0 is the base (blank) signal before introducing the foulant, and ΔS is the recorded difference in signal before and after introduction of the foulant:

$$\Gamma = Q_f \cdot \frac{\Delta S}{S_0} \qquad (1)$$

The value of Q_f depends on the thickness and refractive index, which were determined by ellipsometry. Refractive index increments dn/dc of 0.185, 0.128, and 0.172 $ml.g^{-1}$ were used for BSA, dextrin, and tannin respectively [16,34]. All the experiments were carried out at least in duplicate, using a flow rate of 0.8-1.2 $ml \cdot min^{-1}$. The increase in signal in the first 60 s was used to determine the initial adsorption rate by using the 5-layer matrix model as previously described. This measure is indicative for the kinetics of the adsorption process (see Figure 1B for a schematic impression of the reflectometry experiment and the various parameters that were derived from it).

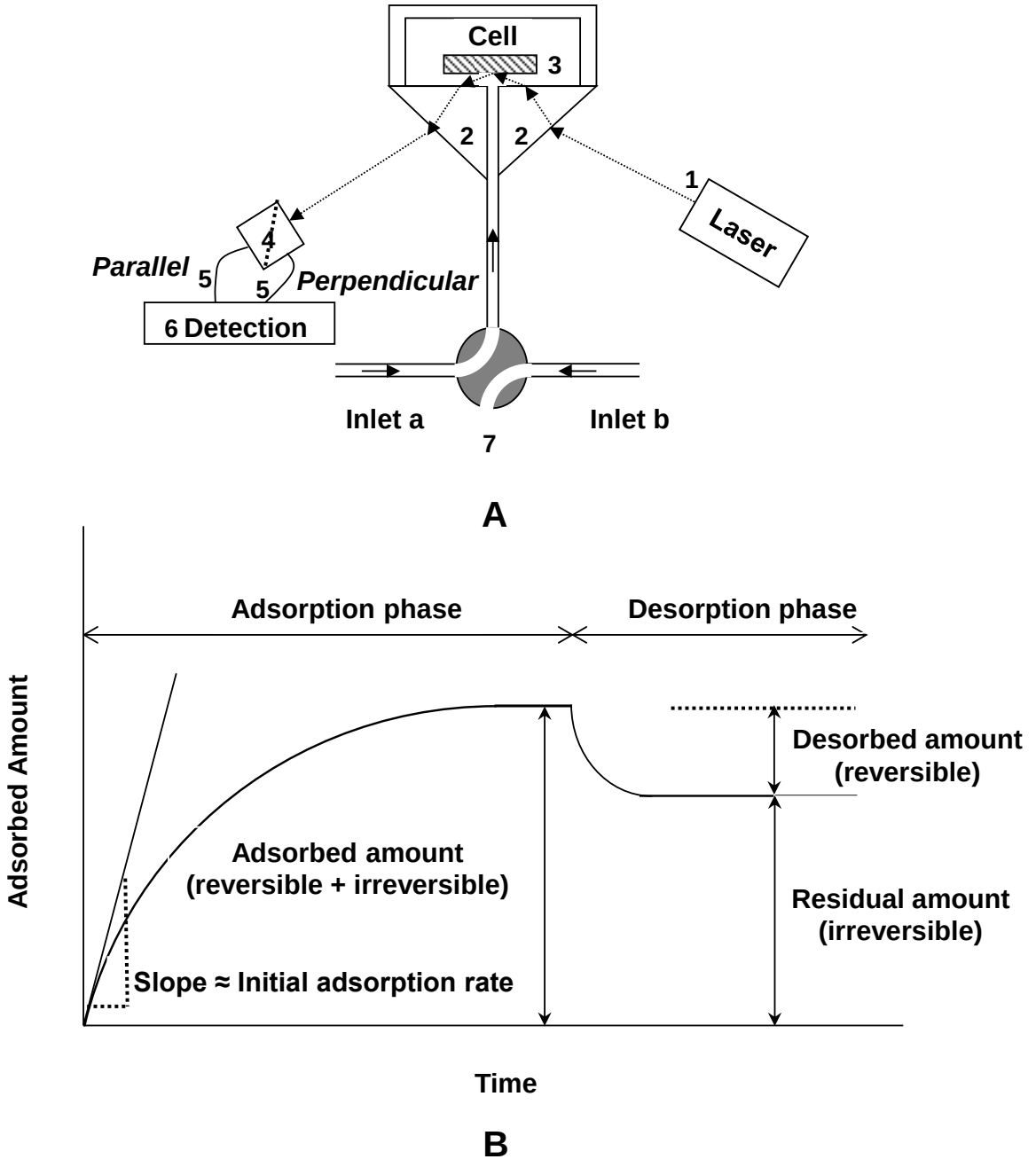


Figure 1. A) Schematic representation of the fixed angle reflectometer, consisting of (1) laser beam, (2) glass prism, (3) sample, (4) beam splitter, (5) photodiodes, (6) detection unit. The actual inlet consists of two (7) 4-way valves (only 1 is drawn here), in order to allow sample to be exposed to up to 3 liquids. **B**) Schematic representation of the reflectometer output.

2.8. Homopolymer Adsorption

Although the enzyme covalently binds the phenolic acid substrate compounds to the surface, homopolymer is also formed in solution, which can then physisorb to the surface. To investigate this, the reaction of phenolic acid substrate and enzyme was carried out as

described before in 40 ml, but now without the PES surfaces present. The reaction time was 2 h and 24 h for 4-hydroxybenzoic acid (28.8 mM), and 7 min, 2 h and 24 h for gallic acid (4.8 mM, and with 24 h reaction time also at 28.8 mM). The enzyme was inactivated by adding 5 ml of 0.1 M NaOH while stirring for 10 min. After re-adjusting the pH to 5 with concentrated HCl, the homopolymer solutions were diluted 10× with Milli-Q water in order to use them in the reflectometer. The refractive index increment d*n*/d*c* of tannin (0.172 $ml.g^{-1}$) was used for the homopolymer, since they are structurally quite similar.

3. Results and Discussion

The research described in this chapter relates to the repellence of foulants (BSA, dextrin, and tannin) by PES model surfaces, modified by phenolic acids by the action of laccase. First, the results that were obtained for the two substrates (4-hydroxybenzoic acid and gallic acid) separately will be discussed, followed by an overview in the overall outlook section. The choice of the reaction conditions are based on previous research [26], in which we evaluated the effect of BSA on 'real' membranes through flux analysis and weight increase. The reaction conditions that were used to modify the model surfaces can be found in the appendix (Tables A1 and A3), both for 4-hydroxybenzoic acid and gallic acid.

3.1. Model Surfaces Modified with 4-Hydroxybenzoic Acid

The unmodified PES surface (see Table 1; modification conditions of the different surfaces are given in appendix A) shows a BSA adsorption of around 2 $mg \cdot m^{-2}$, most of which is irreversibly bound (1.8 $mg \cdot m^{-2}$ remains adsorbed after desorption). The modified surfaces show a lower protein adsorption and in some cases better reversibility (max. 50%). Interestingly enough, the surfaces that were most protein repellent, do not correspond with the most hydrophilic surfaces (low contact angle) as is often assumed to be the case. This will be discussed in more detail in the AFM section (*vide infra*).

Based on the results in Table 1, modification conditions used for surface number 1 (2 h modification using 28.8 mM 4-hydroxybenzoic acid, 0.5 $U \cdot ml^{-1}$ enzyme, at 25 °C and pH 5) were identified as suitable for further research.

Table 1. Adsorption and residual amount after desorption of BSA, contact angle and thickness of modification layer for different model surfaces modified with 4-hydroxybenzoic acid; details on the modification conditions are given in the appendix. ***The bold italic entry 1 is studied in more detail later on.***

Surface number	Thickness of modification layer (nm)	Contact angle (°)	BSA adsorbed amount ($mg \cdot m^{-2}$)	BSA residual amount ($mg \cdot m^{-2}$)
0	Blank	78.9 ± 1.0	2.0 ± 0.25	1.8 ± 0.04
1	***6.3 ± 0.14***	***63.2 ± 1.2***	***0.8 ± 0.15***	***0.4 ± 0.07***
2	4.7 ± 0.55	51.8 ± 3.2	1.3 ± 0.19	1.2 ± 0.22
3	4.7 ± 0.50	63.8 ± 1.5	1.8 ± 0.03	1.5 ± 0.13
4	14.8 ± 0.14	56.8 ± 1.6	1.3 ± 0.15	1.0 ± 0.19
5	6.9 ± 1.00	58.9 ± 0.9	1.1 ± 0.14	0.8 ± 0.10
6	14.4 ± 0.90	52.1 ± 1.7	1.4 ± 0.05	1.2 ± 0.02
7	3.3 ± 0.71	62.8 ± 1.0	0.7 ± 0.02	0.5 ± 0.03

As a next step the modification time was varied. Figure 2A shows the modification layer thickness and resulting contact angle. During the first two hours of modification the modification layer grows fast, thereby lowering the contact angles. The contact angle decreases with increasing modification time until 4 h and remains more or less constant afterwards, while the layer thickness steadily increases with increasing modification time and grows to 15 nm (determined in dry state) after 24 h modification time. Figure 2B shows BSA adsorption and desorption behavior on the modified surfaces. Trend lines were added to guide the eye in all figures. Figure 2B shows for short modification times a reduction in the amount of BSA adsorbed. For longer reaction times, the layer grows more slowly, while the contact angle remains more or less on the same level. At the same time, the amount of BSA that can adsorb to the surface increases again.

The fact that the layer thickness increases only slowly may indicate that the reaction also takes place within the layer, *e.g.* between adjacent grafted chains and/or by the introduction of branches. This would not lead to a thicker layer, but would lead to a more condensed structure of the layer, which could explain the increased BSA adsorption after long modification times [25,26]. The effect of dehydration of the modification layer should also be taken into account, because it may result in an irreversible collapse of the modification layer, leading to only a small increase in the thickness. The effect of surface modification on the adsorption of BSA is visible in the initial adsorption *rate* as depicted in Figure 3. For a blank PES surface the initial adsorption rate is 9.4 ± 0.1 $\mu g \cdot m^{-2} \cdot s^{-1}$, which decreases with a factor of approximately three, to 3.4 ± 0.3 $\mu g \cdot m^{-2} \cdot s^{-1}$ after 2 h of modification. Upon a further prolonged modification, both the adsorbed amounts of BSA and the adsorption rate seem to increase again. Interestingly,

the data points at 4 h and 24 h have the same contact angle, but the adsorption rate increased from 5.5 ± 0.1 to 10.2 ± 1.2 $\mu g \cdot m^{-2} \cdot s^{-1}$ with a slight difference in the final adsorbed amount of BSA. This indicates that, next to a terminal parameter as the hydrophilicity as obtained via the water contact angle, the internal structure of the modification layer is also of significant importance [17,26].

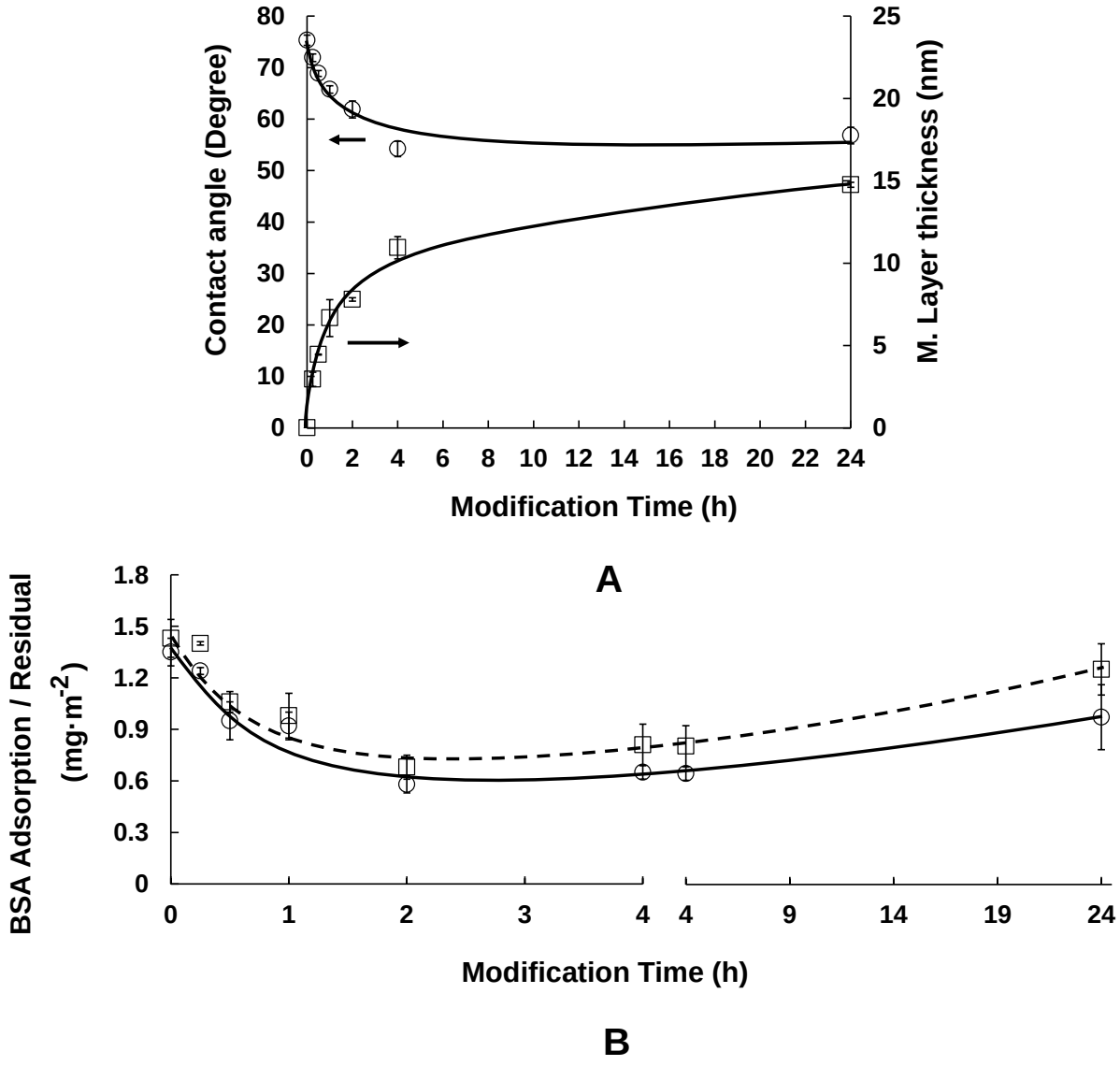


Figure 2. A) Contact angle and modification layer thickness of model PES surfaces, as function of modification time, and **B**) Adsorption (dashed trend line for the square symbols) and residual amount (solid trend line for the circular symbols) of BSA [0.1 $g \cdot l^{-1}$, 10 mM phosphate buffer, pH 7] onto modified PES model surfaces. All were modified with 28.8 mM 4-hydroxybenzoic acid, 0.5 $U \cdot ml^{-1}$ enzyme, at pH 5, and 25 °C.

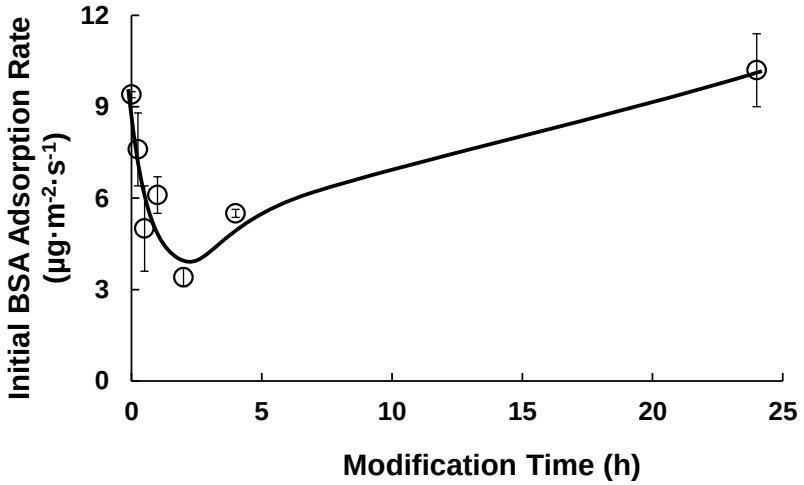


Figure 3. Initial BSA adsorption rate measured on model PES surfaces modified with 28.8 mM 4-hydroxybenzoic acid, 0.5 U·ml^{-1} enzyme, at pH 5, and 25 °C as function of modification time.

AFM experiments carried out with half-modified PES model surfaces (*i.e.*, half of the surface is used as a blank and the other half is modified, see Figure 4), were used to study the morphology of the modification layer. 4-Hydroxybenzoic acid can couple to the PES surface forming grafted oligomers or polymers [25,26]. This is reflected in the roughness of the surface: the RMS height differences increased from 0.7 nm for the blank surface to 2 nm for the modified one.

The half-modified model PES surfaces were also used to visualize the differences in protein adsorption. On the blank surface, white dots appear upon exposure to protein (images E and G, in Figure 4), and the roughness increases considerably from 1.1 to 3.8 nm. On the modified surface, it was difficult to differentiate between the BSA and the grafted oligomers/polymers because the modification itself also leads to the formation of white dots (image F). Furthermore, the RMS before and after adsorption was very similar (1.5 versus 1.0 nm, images F and H). However, the large white patches of protein aggregates that are clearly visible on the blank PES surface (G) were not found on the modified PES surface (H). This in combination with the noted difference in roughness between modified and unmodified PES surface after exposure to BSA, is an indication that the modification layer does lower the adsorbed amount of BSA, as was found with reflectometry.

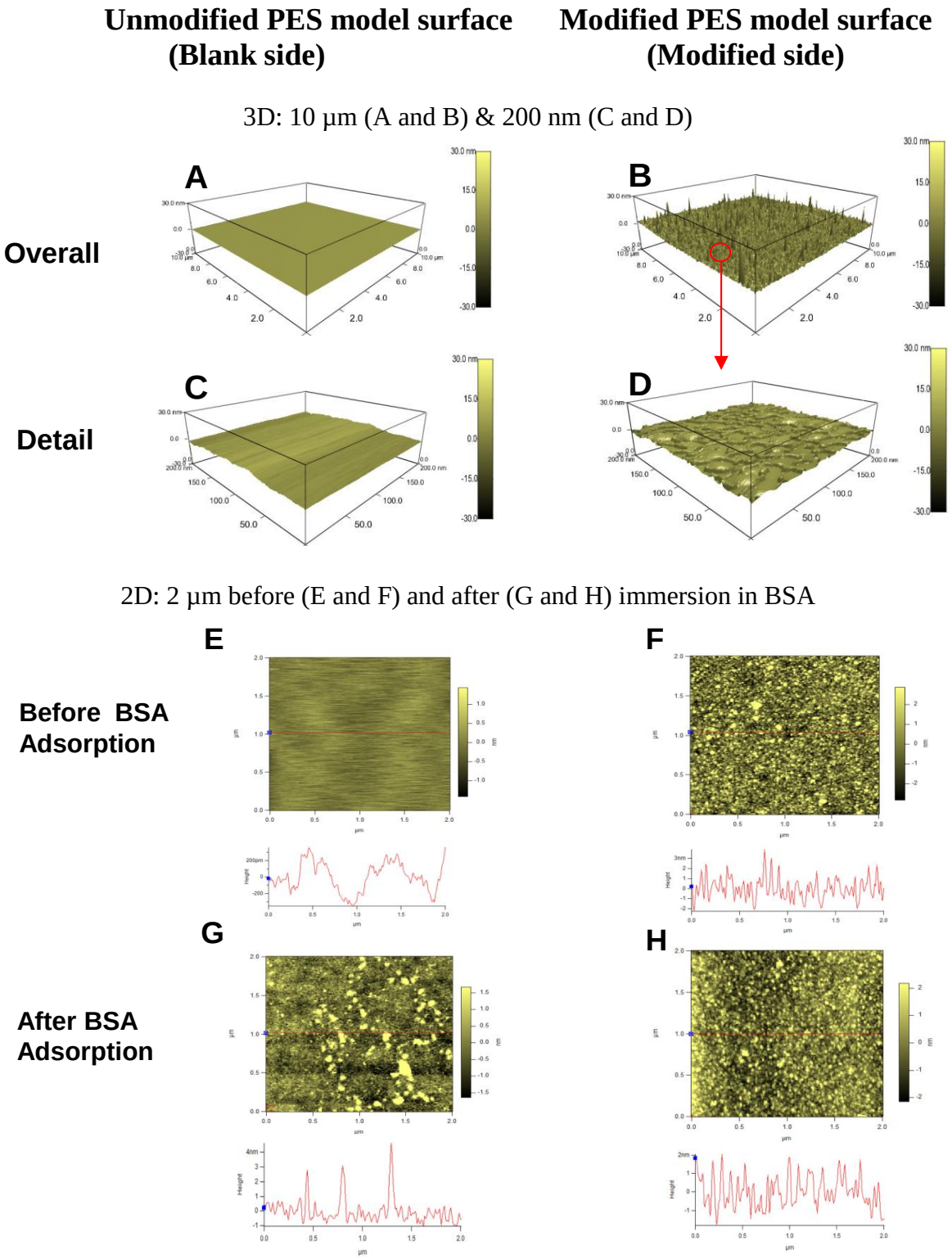


Figure 4. AFM images and height profiles of half-modified PES model surface (both unmodified and modified model PES surfaces are on the same slide) prepared at 28.8 mM 4-hydroxybenzoic acid A, 2 h modification time, 0.5 U·ml^{-1} laccase, 25 °C, and pH 5. 3D: blank side [10 µm (**A**) and 200 nm (**C**)], modified side [10 µm (**B**) and 200 nm (**D**)]. 2D; 2 µm: blank side before (**E**) and after (**G**) immersion in BSA solution, modified side before (**F**) and after (**H**) immersion in BSA solution.

The adsorption of dextrin and tannin (model foulants for polysaccharides and polyphenols) shows similar characteristics to that of BSA adsorption (see Figure 5). Dextrin adsorption is sharply reduced from 0.71 mg·m^{-2} down to 0.03 mg·m^{-2} after 2 h of reaction time. After that the amount adsorbed remains low, though the same increasing trend is visible as was seen with BSA. Tannins initially adsorbed much faster, but are rinsed off easily. The development of the residual amount left after rinsing is again similar to that observed for BSA: a sharp reduction at modification times up to 2 h, and then a gradual increase again.

Once more, this general behavior may well be related to the internal structure of the layer. In the first 2 h, the layer quickly grows, but after that the growth is much slower. Thus, the initial layer will be built up by grafting onto the surface, while at later stages much of the reaction may take place within the layer (*e.g.*, leading to more cross-linking and/or branching and/or collapse of the polymer chains).

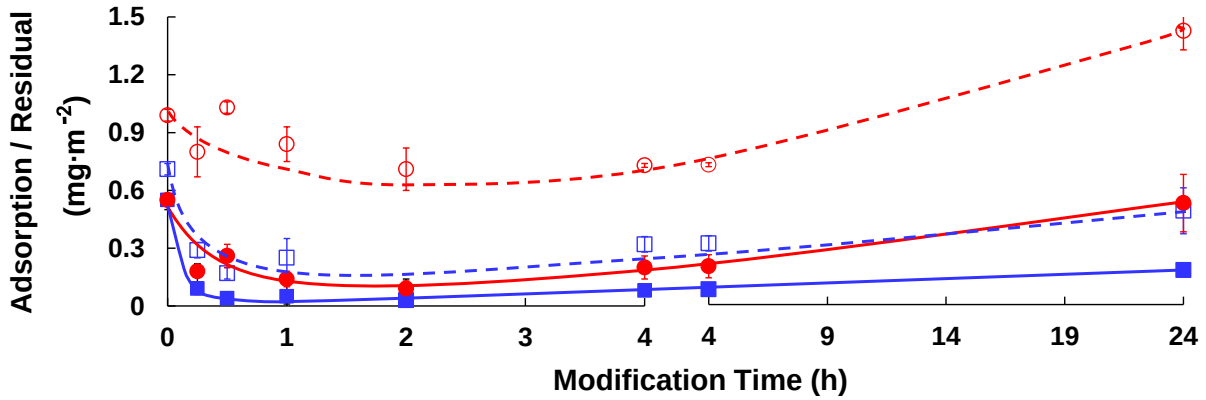


Figure 5. Adsorption (dashed trend line) and residual amount (solid trend line) of dextrin (blue square symbols) and tannin (red circular symbols) (both 0.2 g·l^{-1}) in 10 mM phosphate buffer, pH 7, on modified model PES surfaces as function of modification time [28.8 mM 4-hydroxybenzoic acid, 0.5 U·ml^{-1} enzyme, pH 5, and 25 °C].

In addition, there is also polymerization in the bulk, leading to 4-hydroxybenzoic acid homopolymer in solution, which may then be physisorbed to the model surface. This effect may well disturb our measurements, and therefore we investigated this separately. By only adding the PES model surface after the polymerization, surface grafting can be avoided. Table 2 shows that indeed homopolymer is formed, which adsorbs to the unmodified surface as soon as it is available. As is to be expected with polymer adsorption (usually through multiple adsorption points along the polymeric chain), it is not rinsed off. However, it should be noted that the modified surface hardly shows any adsorption of the homopolymer, and of what is adsorbed most can be removed.

Table 2. Adsorption and residual amount after desorption of 4-hydroxybenzoic acid homopolymer on blank and PES model surface modified for 2 h with 28.8 mM 4-hydroxybenzoic acid, 0.5 U·ml^{-1} laccase, at pH 5 (0.1 M sodium acetate buffer), and 25 °C.

Reaction time for homopolymer formation (h)	Blank PES model surface		Modified PES model surface [2 h modification time]	
	Homopolymer adsorbed amount mg·m^{-2}	Homopolymer residual amount mg·m^{-2}	Homopolymer adsorbed amount mg·m^{-2}	Homopolymer residual amount mg·m^{-2}
2	1.0 ± 0.2	0.9 ± 0.2	0.08 ± 0.03	0.03 ± 0.03
24	1.4 ± 0.1	1.3 ± 0.1	0.02 ± 0.01	0.00 ± 0.00

3.2. Model Surfaces Modified with Gallic Acid

As was the case for 4-hydroxybenzoic acid, all surfaces modified with gallic acid showed less protein adsorption than the blank model surface (Table 3). There is once more no obvious relation between BSA adsorption and the contact angle of the modified model surfaces, which again indicates that hydrophilicity is not the only factor involved. In general, the modification process proceeds faster with gallic acid than with 4-hydroxybenzoic acid. Although this is a positive aspect, homopolymer formation also takes place more quickly. We therefore decided to focus on short reaction times and low substrate concentrations, as was used for surface number 2 (italic bold data in Table 3: 4.8 mM gallic acid, 0.5 U·ml^{-1} enzyme, pH 5, and 25 °C).

Table 3. Adsorption and residual amount after desorption of BSA, contact angle and thickness of modification layer for different reaction conditions during modification with gallic acid; details on the reaction conditions are given in the appendix. ***The bold italic bold entry 2 is investigated in more detail later on.***

Surface number	Thickness of modification layer (nm)	Contact angle (°)	BSA adsorbed amount (mg·m^{-2})	BSA residual amount (mg·m^{-2})
0	Blank	78.9 ± 1.0	2.1 ± 0.12	1.8 ± 0.04
1	5.5 ± 0.91	69.7 ± 1.3	0.8 ± 0.01	0.7 ± 0.03
2	***7.6 ± 0.75***	***64.2 ± 0.6***	***0.5 ± 0.14***	***0.3 ± 0.03***
3	9.5 ± 1.00	67.6 ± 0.8	0.9 ± 0.13	0.8 ± 0.08
4	4.6 ± 0.41	64.4 ± 1.3	1.2 ± 0.17	0.9 ± 0.08
5	10.2 ± 0.76	70.9 ± 1.0	0.8 ± 0.04	0.7 ± 0.04
6	10.3 ± 0.58	62.8 ± 0.6	0.8 ± 0.09	0.7 ± 0.08

In Figure 6, the adsorbed amount of BSA is shown for surfaces obtained after different modification times. There is a minimum in adsorption on surfaces that have been subjected to around 7 min of modification conditions. Most of the adsorption is irreversible, as rinsing does not lead to much desorption. The initial adsorption rate (Figure 7), follows the same trend as the total amount of adsorption with a minimum adsorption rate around 7 min.

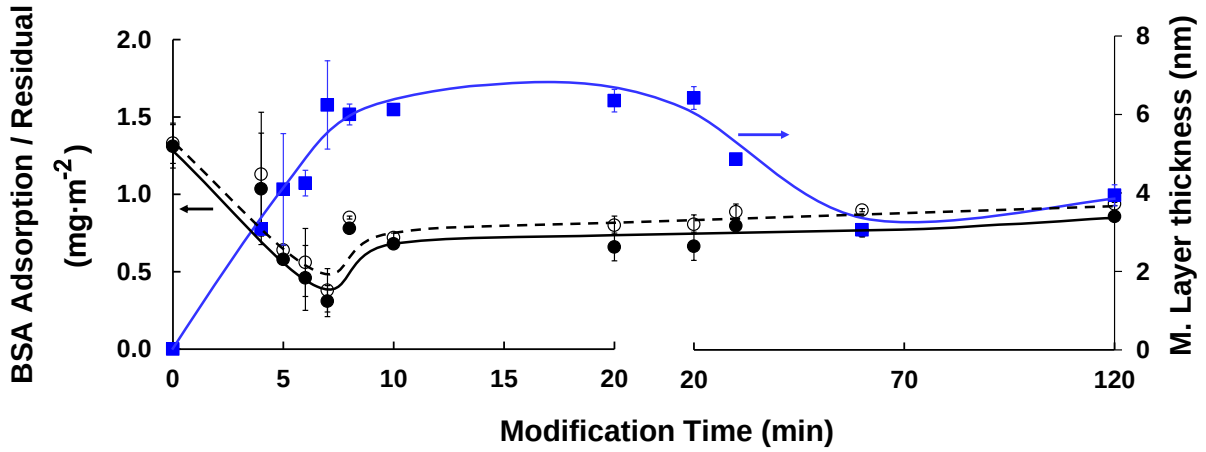


Figure 6. Effect of modification time on BSA adsorption (dashed black trend line) and residual amount after desorption (solid black trend line) on modified PES model surfaces with gallic acid, and thickness of the poly-gallic acid modification layer (nm) (solid blue trend line for the square symbols). [4.8 mM gallic acid, 0.5 U·ml^{-1} enzyme, pH 5, and 25 °C].

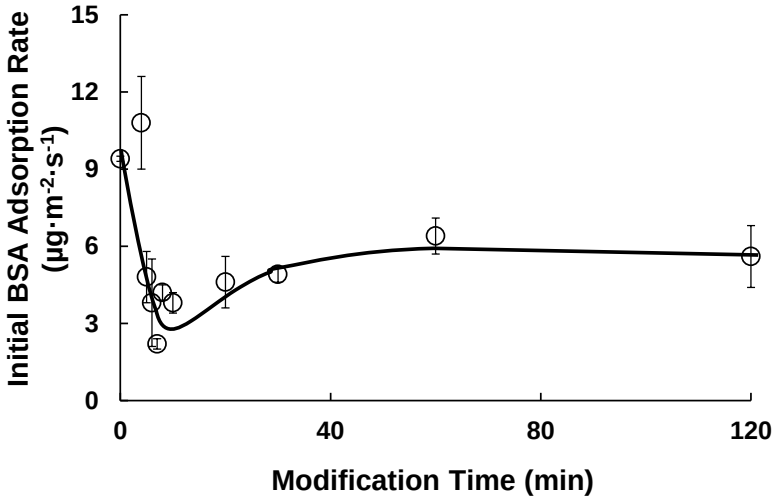
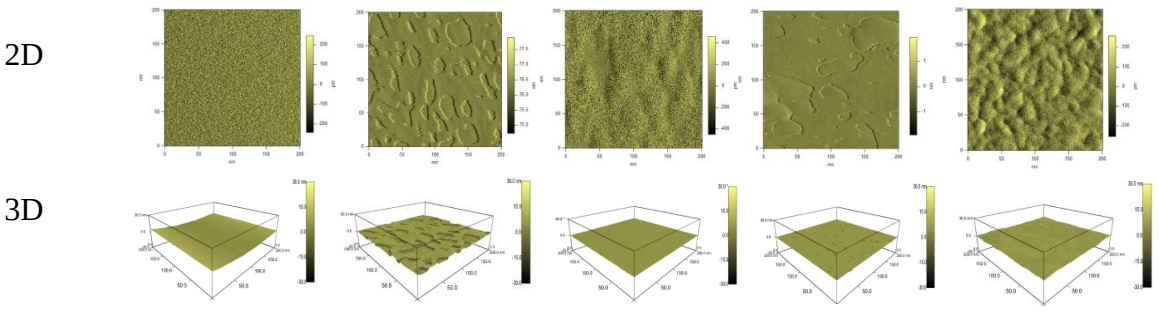


Figure 7. Initial BSA adsorption rate measured on PES model surfaces modified with 4.8 mM gallic acid, 0.5 U·ml^{-1} enzyme, at pH 5, and 25 °C as function of modification time.

Unlike the modification with 4-hydroxybenzoic acid, the layer thickness after 7 min modification time first levels off, and after 20 min starts to decrease again (see Figure 6). This

may well be due to reactions taking place mostly within the modification layer, leading to crosslinks. IRRAS analyses showed characteristic bands of OH moieties at short modification times [25], but these bands were hardly visible anymore upon prolonged modification. This is indicative for the formation of a cross-linked layer. Moreover, the sulfur signal in XPS analysis [26], which becomes appreciably smaller while the layer thickness showed only a marginal increase, provides an extra argument for cross-linking. The cross-linked modification layer loses its protein-repellent properties, as is further illustrated in the AFM section.

Figure 8 shows that the morphology of the modification layer is different from that obtained with 4-hydroxybenzoic acid. The grafted gallic acid appears as small islands after 7 min modification time, which then grow together and become denser and rougher upon longer reaction times. Notice that the layer thickness for 10 and 20 min is very similar (Figure 6), while it is reproducibly thinner at 30 min but looks as a corrugated dense layer. This may be due to excessive cross-linking. Further, it is interesting to note that the RMS roughness of the blank PES surface is much higher after BSA adsorption than for any modified PES surface.



Modification time (min)	Blank	7	10	20	30
Layer thickness (nm)		7	6	6	5
RMS (nm) before BSA adsorption	0.3	2.6	1.3	1.7	0.7
RMS (nm) after BSA adsorption	5.2	1.1	0.6	1.9	1.0

Figure 8. AFM images (2D and 3D), average modification layer thickness, and RMS value before and after BSA adsorption onto blank and gallic acid modified PES model surfaces [4.8 mM gallic acid, 0.5 $U \cdot ml^{-1}$ enzyme, at pH 5, and 25 °C] obtained after 7, 10, 20, and 30 min modification time.

Figure 9 shows that gallic acid modification does yield less adsorption of tannin and dextrin compared to the blank PES model surfaces, but the observed decrease is much smaller as compared to the adsorption of BSA. In case of tannin, the difference between initially adsorbed amount and residual amount is rather large, as was the case for 4-hydroxybenzoic acid, implying that any adsorbed tannin is only weakly adsorbed.

Also for gallic acid, the adsorption of homopolymer was investigated. Again (see Table 4), the homopolymer attached well on the blank PES model surfaces, which was almost completely avoided by modification. The only exception is the modification layer formed at high substrate concentration and modification time, onto which the homopolymer adheres readily, though reversibly.

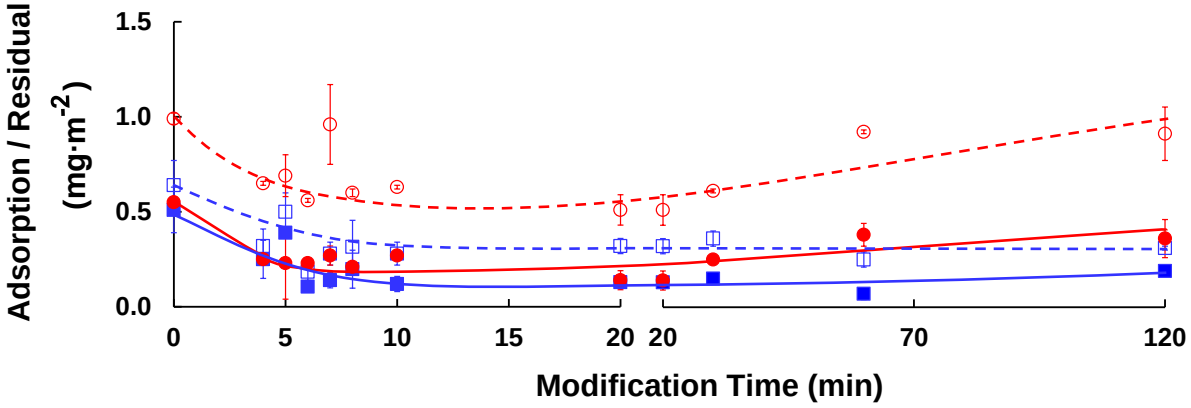


Figure 9. Effect of modification time on dextrin (square symbols) adsorption (dashed blue trend line), and residual amount after desorption (solid blue trend line), tannin (circular symbols) adsorption (dashed red trend line), and residual amount after desorption (solid red trend line) on PES model surfaces [4.8 mM gallic acid, 0.5 U·ml^{-1} enzyme, pH 5, and 25 °C].

Table 4. Adsorbed and residual amount of homopolymer on blank and modified PES model surface. 4.8 mM gallic acid, 0.5 U·ml^{-1} laccase, pH 5 (0.1 M sodium acetate buffer), and 25 °C.

Reaction time for homopolymer formation	Blank PES model surface		Modified PES model surface [7 min modification time]	
	Homopolymer adsorbed amount mg.m^{-2}	Homopolymer residual amount mg·m^{-2}	Homopolymer adsorbed amount mg.m^{-2}	Homopolymer residual amount mg·m^{-2}
7 min	0.8 ± 0.13	0.7 ± 0.15	0.01 ± 0.01	0.00 ± 0.00
2 h	0.6 ± 0.06	0.6 ± 0.02	0.05 ± 0.01	0.02 ± 0.01
24 h	0.5 ± 0.05	0.4 ± 0.03	0.01 ± 0.01	0.01 ± 0.00
28.8 mM gallic acid + 24 h	1.3 ± 0.23	1.2 ± 0.27	1.7*	0.05*

*only one slide was investigated

4. General Discussion

The adsorption of BSA, dextrin and tannin to the PES surface was reduced with laccase-catalyzed modification using both substrates (4-hydroxybenzoic acid and gallic acid). However, the extent of foulant repellence differs depending on the structure of the substrate and the reaction conditions. At short modification times, the adsorption of the three foulants was reduced, whereas at longer modification times, the adsorption increases again for both substrates.

We found that the more hydrophilic surfaces were not necessarily best in protein repellence; apparently, the internal polymer structure adds to the created effects. This is in good agreement with recently published research about the design of novel antifouling surfaces using surface-initiated atom transfer radical polymerization (SI-ATRP) to grow poly[N-(2-hydroxypropyl) methacrylamide] brushes on a gold-coated substrate [35]. This brush structure shows decreased adsorption of proteins from blood plasma to a level below the detection limit of surface plasmon resonance (SPR, 0.03 $ng.cm^{-2}$), in spite of its only moderate wettability (advancing and receding θ are 40° and 21°, respectively, 17 nm layer thickness). This research and our results show that there is no direct relationship between fouling of a surface and its hydrophilicity, which is in contrast to the currently proposed theory for the design of protein-resistant surfaces. It is clear that the process of protein adsorption to surfaces is still not well understood.

All the results suggest that at short reaction times, the surface becomes grafted, leading to a thin layer that is more hydrophilic than PES. This layer is effective in reducing any strong adsorption of protein, polysaccharide and polyphenol. At longer reaction times, other effects become more prominent. One effect is that as more material is grafted on the membrane surface, the enzyme and/or enzyme-generated radicals will also be active within the layer, leading to cross-linking between adjacent chains (especially for gallic acid), branching and/or collapse of the chains. This will lead to a modifying layer that is less swollen and denser.

In addition, further grafting may lead to a more irregular surface of the modification layer. Any protrusions from the film will have faster access to the reactants in the bulk, and thus will grow somewhat faster than their surroundings. This will lead to the protrusions to grow and become more prominent. This well-known effect ultimately leads to rougher modification layers, which then will increase the adsorption onto the film, since it gives more anchoring points for the adsorbents to attach.

A third effect is the formation of homopolymer in the solution, which can then adsorb to the surface. This will also lead to a rougher surface and therefore to a stronger adsorption of other components. However, this effect is not very likely to have played a major role in our experiments, given the low affinity of the homopolymer for modified surfaces.

The reactivity of gallic acid is much higher than that of 4-hydroxybenzoic acid, given the extra two OH groups it contains. This allows for faster modification with a rather different polymer structure, and at the same time homopolymer formation takes place much faster, which may lead to inefficient use of the monomer for the conditions chosen here. However, with careful choice of the (very mild) reaction conditions, the surface structure can be well controlled.

5. Conclusions

Enzyme-catalyzed modified poly(ethersulfone) (PES) model surfaces show a reduction in the adsorption of BSA, dextrin and tannin that is dependent on the internal structure of the formed polymer layer. PES surfaces can be effectively modified by phenolic compounds such as gallic acid and 4-hydroxybenzoic acid, and both types of modifying layer reduce the adsorption of proteins, polysaccharides, and polyphenols. The extent of the reduction in adsorption can be influenced with the modification conditions.

Short modification times gave strongly reduced adsorption; at longer modification times this antifouling effect was diminished. This is probably due to an interplay of (fast) surface grafting, which is effective against adsorption, and (slower) intra-layer cross-linking, and/or branching, and/or collapse of the polymer chains. The reduction in adsorption is clearly not purely related to the contact angle: also the internal polymer layer structure plays an important role.

With both 4-hydroxybenzoic acid and gallic acid, modified surfaces can be obtained that affect BSA adsorption (and smaller effects were found for dextrin and tannin). Although the efficiency of the modification process is co-determined by homopolymer formation (which leads to inefficient substrate use), the homopolymer as such, especially in case of 4-hydroxybenzoic acid, does not influence the modification layer because of the inherent repelling properties of this layer.

Acknowledgments

The authors would like to thank ISPT for financial support of this research.

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Appendix A. Modification conditions used in Tables 1 and 3; surfaces obtained after treatment given ***in bold italic print*** are used as starting point for experiments explained in detail in the text.

Table A (1). Modification conditions used for 4-hydroxybenzoic acid.

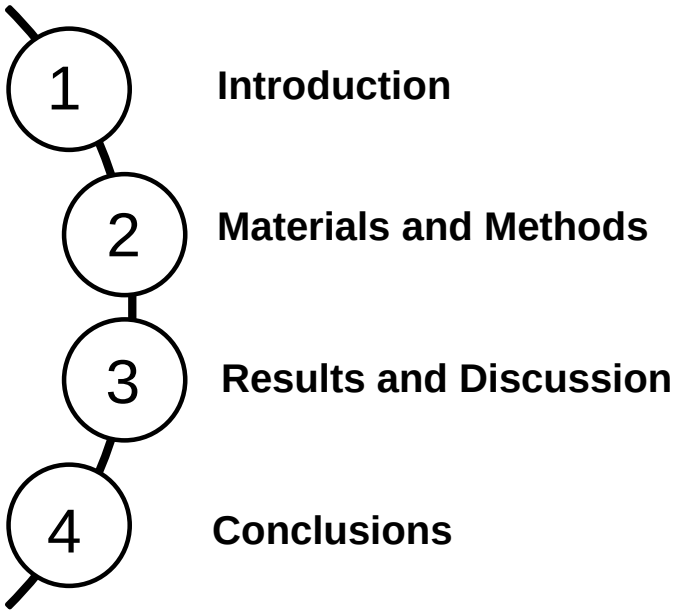
Surface number	Concentration 4-hydroxybenzoic acid (mM)	Concentration laccase ($U \cdot ml^{-1}$)	Modification time (h)	Modification temperature (°C)	pH
0	Blank				
1	***28.8***	***0.5***	***2***	***25***	***5***
2	4.8	1.0	2	25	5
3	4.8	0.5	24	25	5
4	28.8	0.5	24	25	5
5	4.8	0.5	2	55	5
6	28.8	0.5	2	55	5
7	4.8	0.5	2	25	6

Table A (3). Modification conditions used for gallic acid.

Surface number	Concentration gallic acid (mM)	Concentration laccase ($U \cdot ml^{-1}$)	Modification time (h)	Modification temperature (°C)	pH
0	Blank				
1	4.8	0.5	30	25	5
2	***4.8***	***0.5***	***120***	***25***	***5***
3	28.8	0.5	120	25	5
4	4.8	0.75	120	25	5
5	28.8	0.5	120	55	5
6	28.8	0.5	120	25	6

Chapter Six

Listeria Repellence by Modified PES Surfaces



Part of this chapter will be published as:

Listeria monocytogenes Repellence by Enzyme-Catalyzed Modified PES Surfaces, Norhan Nady, Stijn van der Veen, Karin Schroën, Maurice Franssen, Mohamed Mohy Eldin, Tjakko Abee, Han Zuilhof, and Remko Boom.

Listeria monocytogenes Repellence by Enzyme-Catalyzed Modified PES Surfaces

Abstract

In this chapter, we evaluate the effect of the enzyme-catalyzed modification of poly(ethersulfone) (PES) on the adhesion of *Listeria monocytogenes*, which is chosen as a model bacterium. It is known to cause serious problems in both food industries and water treatment.

PES was modified with 4-hydroxybenzoic acid and gallic acid, because the resulting layers showed repellence of various foulants as described in the previous chapters. In addition, PES was modified with ferulic acid, which in literature is claimed to have an antimicrobial effect. Two strains of *Listeria monocytogenes* were applied: the commonly used EGD-e strain and the well-known biofilm forming strain LR-991.

Listeria monocytogenes adhesion and biofilm growth was investigated both under static and dynamic conditions. The surfaces modified with any of the three tested compounds repelled *Listeria* adhesion up to 70% (static conditions) or 95% (dynamic conditions). Biofilm growth was inhibited up to about 70%, depending on temperature (presence/absence of flagella), and the conditions and substrate used for modification.

1. Introduction

Listeria monocytogenes (*L. monocytogenes*) is a gram-positive bacterium. People infected by this bacteria get a disease known as listeriosis [1,2], which can cause miscarriage, newborn infection, dangerous illnesses such as meningitis and septicaemia, and even death. *Listeria* species can attach to all kinds of surfaces including plastics, rubber, stainless steel, glass, etc. [3,4]. This pathogen can survive and grow at a very wide temperature range (below freezing point up to 46 °C), high salinity (grows up to 13% and remains alive up to 30%), and wide pH range (below 5 and up to 9). The reason of survival and growth of these bacteria in such severe conditions is attributed to their ability to adapt themselves to changing environments [5,6].

L. monocytogenes is associated with foods like raw milk, cheese, ice cream, and raw and smoked fish [7,8]. It also occurs in sea, sewage, and river water [9-13]. The factors that affect adhesion of *L. monocytogenes* to surfaces are not completely understood yet. As influential factors bacterial cell surface properties, the properties of the substratum (inert) surface, and the local conditions have been mentioned [5,14]. Although in literature it has widely been suggested that hydrophilic, negatively charged, and smooth surfaces are more effective in reducing the initial adhesion of live cells [15], many researchers have reported that neither initial adhesion of *L. monocytogenes* nor biofilm formation depend on the surface roughness [16]. Also, the effect of surface hydrophilicity on attachment of *Listeria* on polymeric surfaces is limited (less than one order of magnitude) [17]. On the other hand, it is known that growth conditions may be of great influence on cell adhesion. For example, at temperatures below 30 °C *Listeria* cells produce a flagellum, a tail-like projection that is used for locomotion. The flagellum is rich in negatively charged proteins that strongly influence the physicochemical (electrostatic) properties of the microbial cell surface and consequently its adhesion to surfaces [18].

Poly(ethersulfone) (PES) membranes are widely used in the food industry and for water treatment (separation and purification purposes). However, the drawback of this type of membranes is the significant adhesion of proteins and living cells, with a severe reduction in membrane performance (flux and selectivity) as a consequence. The resulting membrane replacement that needs to take place regularly forms the largest operating cost in any membrane separation process [19].

In our previous research [20], we showed that it is possible to covalently link phenolic compounds such as 4-hydroxybenzoic acid and gallic acid via their oxygen atoms to PES

surfaces using laccase from *Trametes versicolor*, an enzyme that can catalyze grafting in aqueous medium under mild conditions. This modification leads to a remarkable suppression of the protein adsorption on both modified 'real membranes' [Chapter 4, 21] and modified laminated PES on a silicon dioxide surface (model PES surfaces) [Chapter 5, 22]. Since protein adsorption is often proposed as an initial step to microorganism adhesion, we decided to test the ability of the modified PES surfaces to resist attachment and biofilm growth by bacteria. In this study *L. monocytogenes* was used as a model microorganism. Two modification conditions using 4-hydroxybenzoic acid and gallic acid, which were shown to significantly reduce protein adsorption [22], were also applied here. In addition, ferulic acid, which has an anti-bacterial action according to literature [23], was used as modifier.

EGD-e [24] and LR-991 [25] strains of *L. monocytogenes* were used, as the former is commonly used in biological studies (the first sequenced strain), while the latter is a well-known biofilm former. *Listeria* attachment and biofilm growth were assessed both under static and dynamic conditions, as described in the materials and methods. The standard plate count method was performed for initial evaluation, whereas fluorescence microscopy was used to support the results.

2. Materials and Methods

2.1. Chemicals

From Sigma-Aldrich were purchased: catechol (>99%), sodium acetate (anhydrous, ≥99%), acetic acid (99.9%), 4-hydroxybenzoic acid (99%), dichloromethane (ACS, stabilized, 99.9%), gallic acid (>97.5%), ferulic acid (99%), potassium chloride (99%) and laccase from *Trametes versicolor* (>20 $U \cdot mg^{-1}$). Poly(ethersulfone) (PES) (Ultrason, E6020P) was obtained from BASF (Ludwigshafen, Germany). 2,2′-Azobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was obtained from Calbiochem. Prime grade 150 mm silicon wafers of type P/B <100> orientation, thickness 660-700 µm, 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, 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 Na_2HPO_4, 2.4 g anhydrous KH_2PO_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 preparation of the model PES surfaces and sterilized reverse osmosis (R.O) water was used throughout the biofouling tests.

2.2. Laccase Assay

Laccase activity was determined with 2,2′-azobis(3-ethylbenzothiazoline-6-sulphonic 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 $U \cdot ml^{-1}$ laccase. Oxidation of ABTS is monitored by following the increase in absorbance at 420 nm ($\varepsilon = 36{,}000\ M^{-1} \cdot cm^{-1}$) [26]. The reaction time taken is 1 min. One unit of laccase activity is defined as the amount of enzyme required to oxidize 1 µmol of ABTS per min at 25 °C.

2.3. Preparation of Modified PES Surfaces

Silicon wafers (with approximately 70 nm silicon dioxide layer) were cut into strips of 1 × 5 cm (static conditions) or 1.5 × 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 % w/w 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 × 5 cm strips) or 33 ml (1.5 × 5.5 cm strips) of 0.1 M sodium acetate buffer containing different concentrations of phenolic acids (substrates/monomers) and laccase. A flow of air was used for mixing and as oxygen source. 4-hydroxybenzoic acid (28 mM, 2 h modification), ferulic acid (4.8 mM, 1 h and 24 h modification), and gallic acid (4.8 mM, 7 min modification) were used as enzyme substrates [20-22]. Common modification conditions were 0.5 $U \cdot ml^{-1}$ enzyme concentration, temperature 23 ± 2 ºC, pH 5 in 0.1 M sodium acetate buffer. 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.

2.4. L. monocytogenes Strains and Culture Conditions

L. monocytogenes strains EGD-e and LR-991 [24,25] 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 or 37 °C.

Overnight-grown cultures were used to inoculate (1%) 25 ml BHI in 50 ml tubes and incubated for 24 h at 30 °C or 37 °C. Cells were harvested by centrifugation at 5000 rpm for 15 min at 20 °C and subsequently re-suspended in 1 ml PBS (attachment) or BHI (biofilm formation).

2.5. L. monocytogenes Attachment and Biofilm Formation

2.5.1. 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 10^9 total cfu). The PES slides were incubated for 2.5 h at room temperature (23 ± 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 or 37 °C for 48 h colonies were enumerated. 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 and the slides were incubated for 24 h.

2.5.2. Dynamic Conditions

Bacterial attachment under dynamic conditions was tested using a flow cell. A 1.5 x 5.5 cm size slide was loaded on the sample support inside the flow cell (sample support size: 1.6 cm width x 5.7 cm length x 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 minutes and subsequently filled with the bacterial suspension. The bacterial suspension was circulated (0.038 $m \cdot s^{-1}$ - 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 enumerated as described for static conditions. Experiments were performed in three independent biological replicates.

2.6. Fluorescence Microscopy

Fluorescence microscopy experiments were performed on a BX41 microscope (Olympus, Zoeterwoude, the Netherlands). Images were acquired using a XC30 camera (Olympus) and Olympus Cell^B software. After washing the modified PES slides twice with PBS, it was placed on a microscope slide (76 x 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).

Statistics. Microsoft Excel was used to apply t-test on the obtained data; differences were considered to be statistically significant at $P < 0.05$.

3. Results and Discussion

The overall purpose of this study was to examine the effect of laccase-catalyzed modification of PES surfaces on the adhesion and biofilm formation of *L. monocytogenes* under both static conditions and dynamic conditions. The obtained results were presented in two ways: the number of bacteria cells per unit area (in log scale) and as percentage of reduction relative to the blank (unmodified) PES surface.

3.1. Static Conditions

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 (*i.e.* flagella are present) was determined and depicted in Figure 1 (white bars; $P < 0.05$ for all samples). The results show approximately 60% reduction in the number of attached *Listeria* cells from both strains relative to the blank surface. The high reduction found on PES surfaces modified by 4-hydroxybenzoic acid (63%, $P < 0.004$) could be attributed to the brush-like structure of the modification layer [22]. Possibly the effect is similar as demonstrated in other studies [27-29], where the brush-like structure of the modifying layer on different surfaces *e.g.* 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 [29].

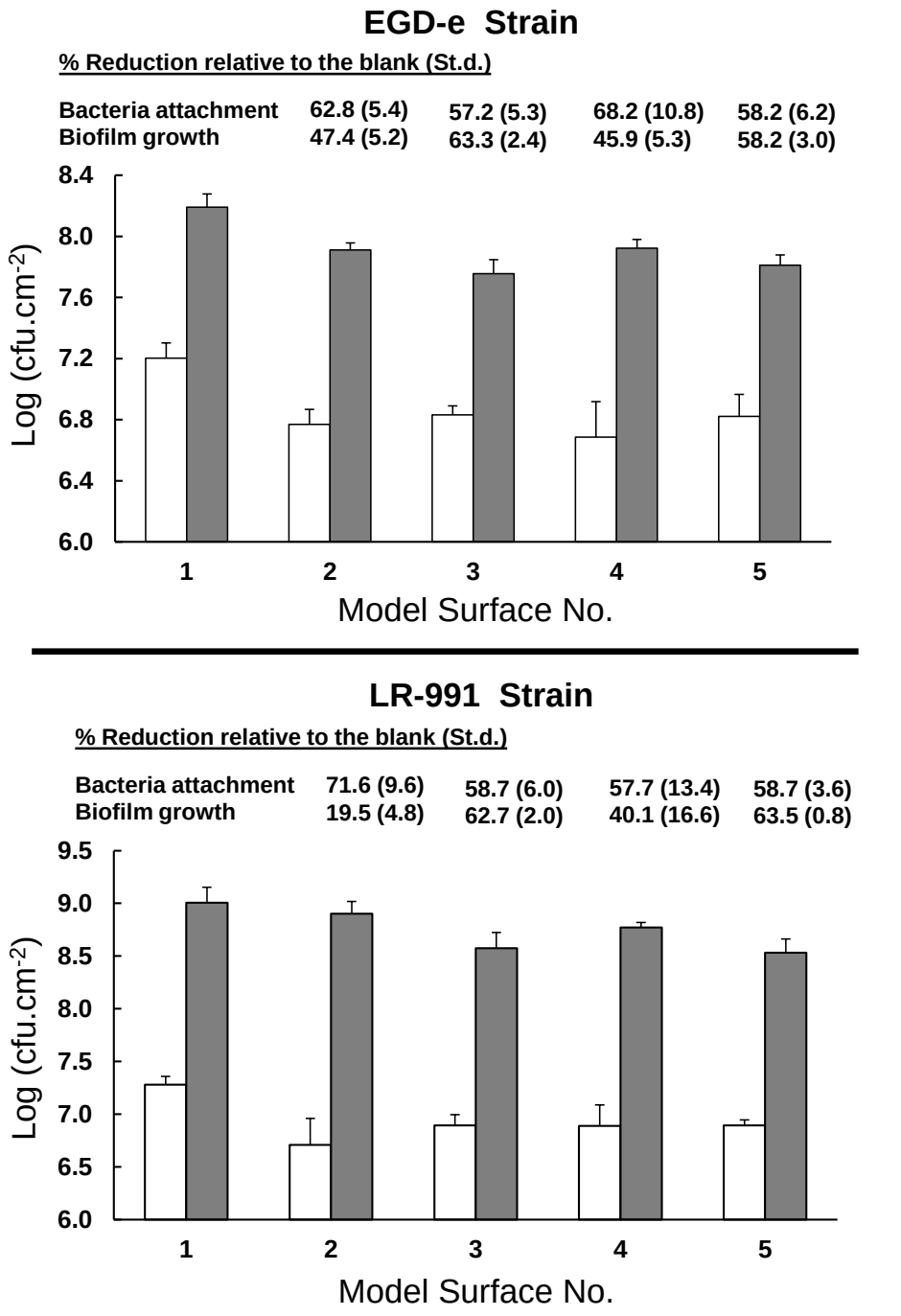


Figure 1. Effect of PES modification on cell adhesion (white bars) and biofilm formation (grey bars) under static conditions, shown as the number of *L. monocytogenes* cells adhering per unit area, and as the percentage of reduction relative to the blank surface. Error bars represent the standard deviation (St.d.) over four model surfaces, using two separate bacterial cultures.

This interpretation is also plausible for gallic acid; at the chosen reaction conditions, a brush-like structure is formed [21,22]. PES surfaces modified with ferulic acid also show inhibition of bacterial adhesion. However, the structure of the layer is still not known and is in need of further investigation. Possibly at short reaction time, a brush type layer is formed that later may cross-link as was the case for gallic acid.

Also under static conditions, biofilm formation of *L. monocytogenes* EGD-e and LR-991 strains grown at 30 °C (flagella are present) on modified surfaces was determined (Figure 1, grey bars; $P < 0.05$ for all samples except the modification using 4-hydroxybenzoic acid with LR-991 strain). The PES surfaces modified with ferulic acid and gallic acid exhibit percentages of reduction close to their percentages of reduction in the attachment test (~60%). This is may be attributed to the same phenomena (steric hindrance) to be involved in both cell adhesion and biofilm formation, however the biofilm formation by the LR-991 strain was hardly affected by modification of PES with 4-hydroxybenzoic acid (19.5%; $P >> 0.05$).

Changing the time that PES surfaces were modified with 4-hydroxybenzoic acid has an effect on the inhibition of biofilm formation for both strains (static conditions), as is visualized in Figure 2.

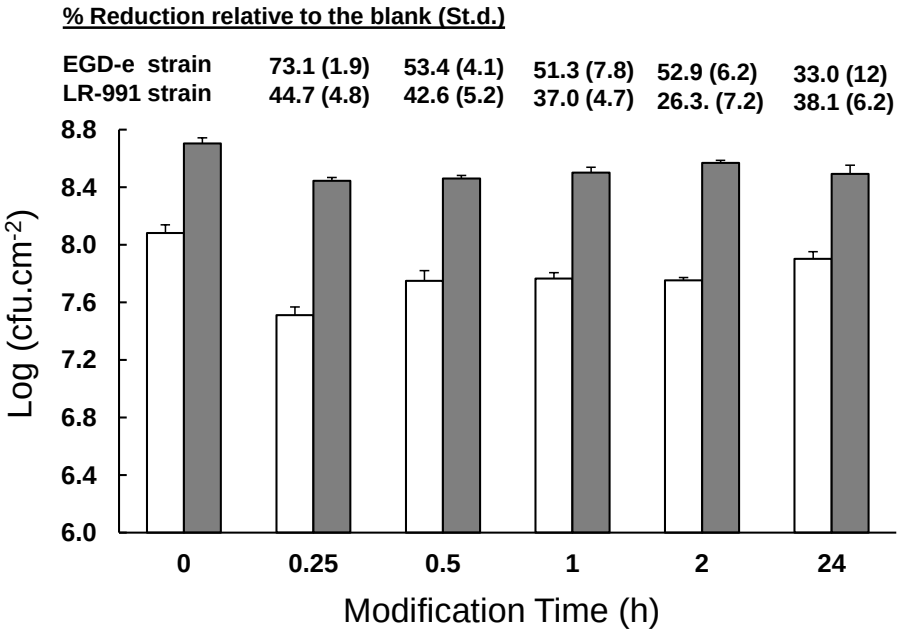


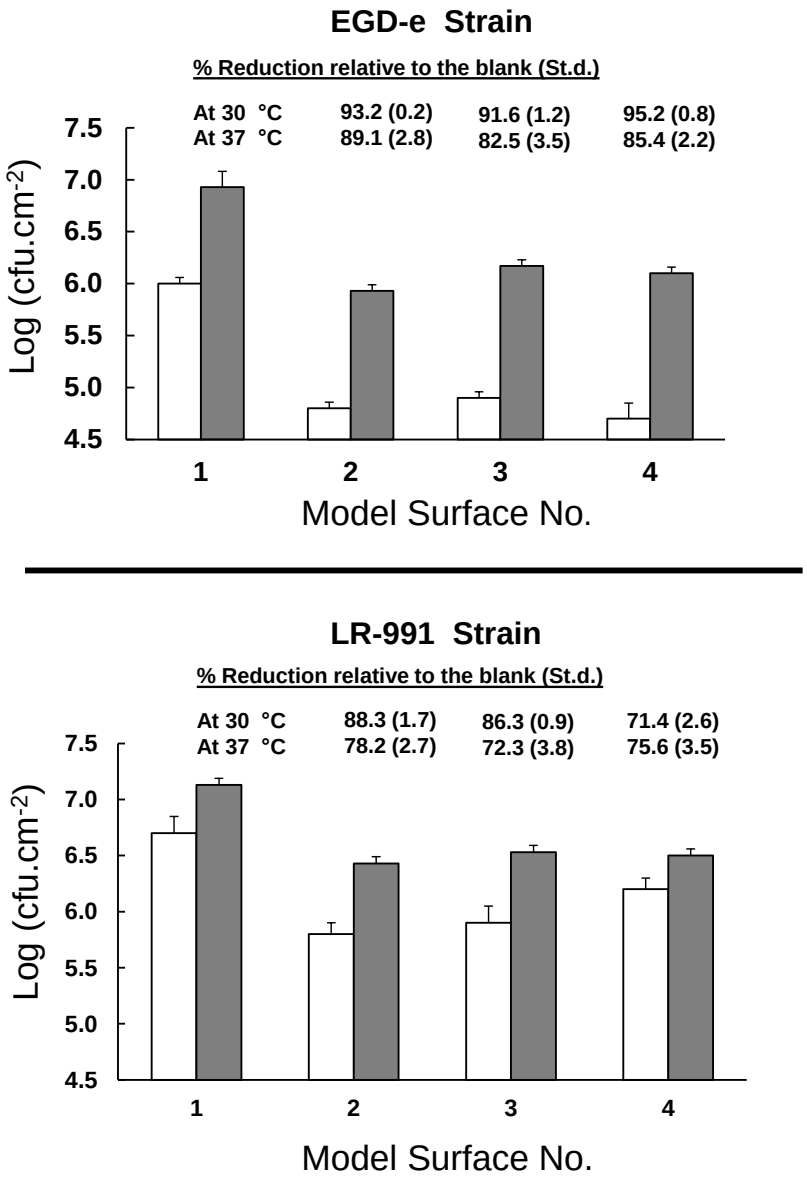
Figure 2. Effect of the modification time of PES surfaces modified with 4-hydroxybenzoic acid on *Listeria* biofilm formation. The number of *L. monocytogenes* cells adhering per unit area and the percentage of reduction relative to the blank surface were determined using static test conditions. White bars: EGD-e strain, grey bars: LR-991 strain. Error bars represent the standard deviation (St.d.) over four model surfaces, using two separate bacterial cultures.

It seems that the percentage of reduction was highest at short modification times. This indicates that the formed structure needs to be accurately tuned to the properties of the bacterial interaction to optimize the antimicrobial effect, similarly as was observed for protein adsorption (see chapter 4 and 5). While this aspect is currently still poorly understood, it displays significant potential to be used effectively against biofilm formation.

From a microbiological point of view, it seems there is no significant difference between the used substrates: all the modified surfaces resist the attachment of bacteria cells and consequently the biofilm formation to some extent. This reduction is not large enough for pathogens because a decimal reduction of at least 2 (ideally 4) is needed here. However, our results may be acceptable for non-pathogenic strains of *Listeria* or other bacteria.

3.2. Dynamic Conditions

The effect of flowing *L. monocytogenes* suspension in PBS over the model surfaces (dynamic conditions, only bacterial attachment was tested) at 30 and 37 °C was studied for both strains; the results are depicted in Figure 3. The data are the output of three surface sets using three different cultures prepared on three different days. Modification of the surfaces led to a reduction of the adhered cells compared to the static measurements, especially at a growth temperature of 30 °C, at which the cells have flagella. The presence of flagella may assist in electrostatic repulsion between the cells and the modification layers, because both of them carry the same groups (COO^-). At 37 °C, the cells do not possess the negatively charged flagella so inhibition of adhesion is less prominent, but still significant. 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 [30]. 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.



1. **Blank**
2. **Modified by 4-hydroxybenzoic acid (28.8 mM + 2 h modification)**
3. **Modified by ferulic acid (4.8 mM + 1 h modification)**
4. **Modified by gallic acid (4.8 mM + 7 min modification)**

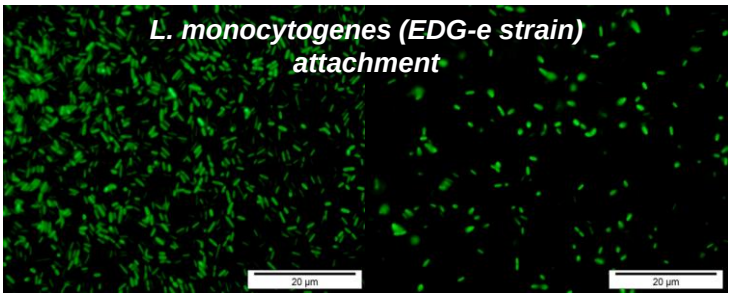
Experimental Conditions: 0.5 U·ml^{-1} enzyme, 25 °C, and pH 5 [0.1 M sodium acetate buffer]

Figure 3. The number of *L. monocytogenes* cells adhering per unit area and percentage of reduction relative to the blank surface was investigated using dynamic testing conditions at 30 (white bars, with flagella) and 37 °C (grey bars, without flagella). Error bars represent the average standard deviation (St.d.) of three surfaces from three separate cultures.

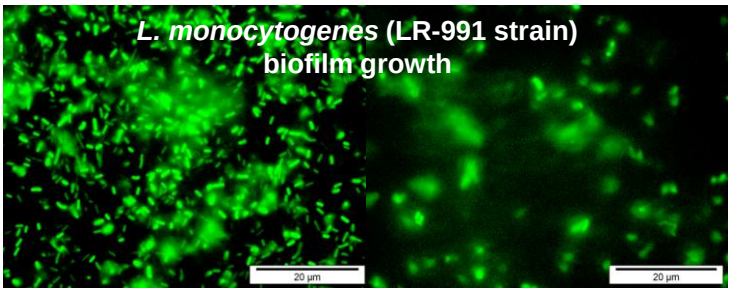
3.3. Fluorescence Imaging

Fluorescence images for two modified surfaces are shown in Figure 4. 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.



Blank PES model surface | PES model surface modified by 4-hydroxybenzoic acid



Blank PES model surface | PES model surface modified by ferulic acid

Figure 4. Fluorescence images of blank and modified model PES surfaces using static testing conditions. The surfaces were modified during 2 h with 4-hydroxybenzoic acid and 1 h with ferulic acid.

4. Conclusions

The obtained results indicate that the enzyme-catalyzed modification of PES surfaces affects *L. monocytogenes* attachment (and consequently the biofilm growth). Depending on the used substrate a reduction of 40-60% is found under static conditions, while this percentage increases to up to 95% under dynamic conditions. Although this reduction is not sufficient for pathogenic bacteria like the used *Listeria* strains, it may be useful for the repellence of non-pathogenic bacteria. Furthermore, we have modified PES with only three different phenolic substrates under a limited number of conditions, so substantial optimization is still possible.

In conclusion, the enzyme-catalyzed modification method presented in this thesis is an interesting eco-friendly method to reduce biofouling on PES membranes by influencing bacterial attachment. This is an important prerequisite for application of such modified

membranes in water treatment as well as in food processing, because the anti-biofouling effects can strongly reduce the replacement costs that are currently a major hurdle.

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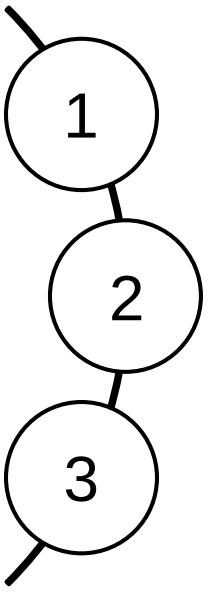
Chapter Seven

General Discussion and Outlook



Current Status

Practical Aspects

Versatility and Future Developments

General Discussion and Outlook

In the following part, the findings presented in the previous chapters are summarized and discussed. From there, a short outlook on practical aspects for industrial application is presented. The chapter is concluded with additional options that may be used to further extend the presented technology.

1. Current Status

In the previous chapters, we have described the discovery and use of an enzyme-catalyzed modification of poly(ethersulfone) (PES) surfaces and membranes. The enzyme laccase was used to covalently bind phenolic acids to PES by C-O linkages. Other monomers can be oxidatively grafted onto the attached monomers, to form oligomers or polymers, which may lead to additional C-O as well as C-C bond formation with concomitant coloration of the surfaces. Figures 1 and 2 schematically show the reactions occurring with 4-hydroxybenzoic acid and gallic acid, respectively (see also Chapter 3 and 4). Due to the presence of three adjacent hydroxyl groups on the aromatic ring of gallic acid, further oxidation of the formed radicals to *o*-quinones may take place inside the enzyme's active site or in solution as shown in Figure 2 (see Chapter 4 for more information).

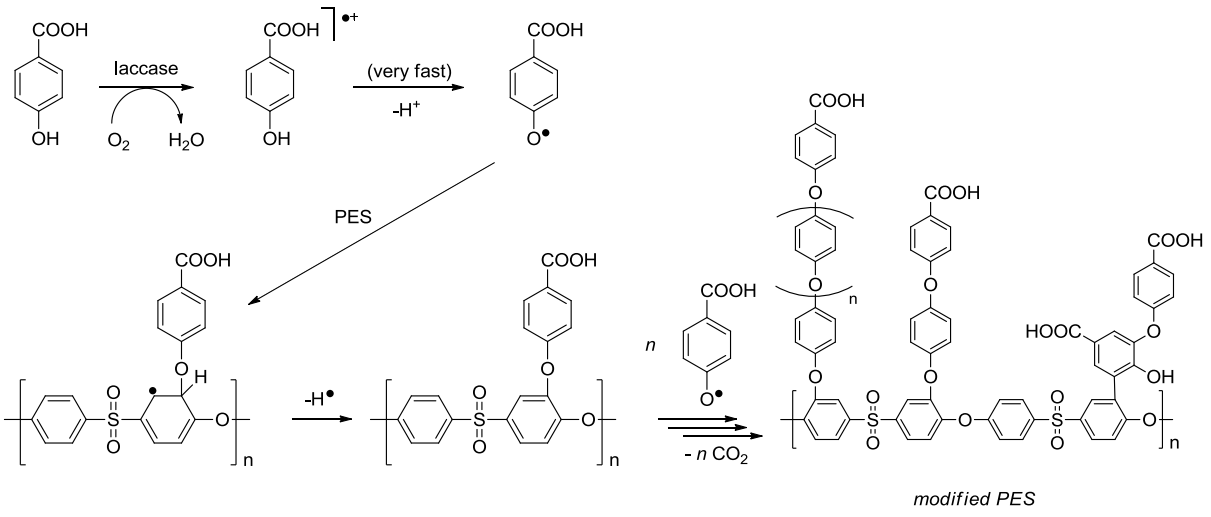


Figure 1. Tentative mechanism for the formation of reactive 4-hydroxybenzoic acid radicals by laccase and grafting of the radicals to PES membranes.

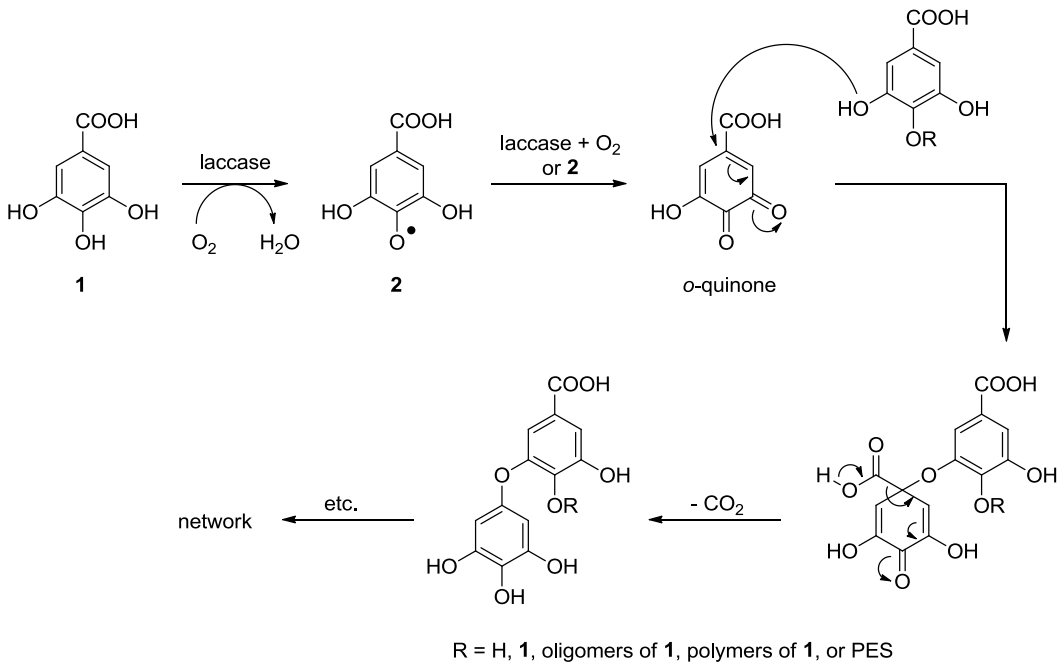


Figure 2. Tentative mechanism for the laccase-mediated formation of an *o*-quinone from gallic acid, and its reaction with gallic acid (derivatives) in solution or with the PES membrane.

The layer structure can be tuned by the modification conditions and the choice of substrate. In general, substrates with more reactive groups (like gallic acid) will lead to denser 3D networks, while molecules with only one hydroxyl group give linear or branched structures, which swell and extend in water to give rise to entropic repulsion as shown in Figure 3 for 4-hydroxybenzoic (4-HBA) and gallic acid (Chapter 4). Besides the surface reactions, monomers can take part in oxidative grafting to other monomers or oligomers to form homopolymers in solution that can adsorb to the membrane surface. This homopolymer is not desirable, because it leads to inefficient substrate use. Furthermore, the short chains of this homopolymer may physisorb to the surface, and if desorption takes place during the filtration process, the permeate and/or the retentate may be contaminated. However, good washing by *e.g.* a water flux after modification should be sufficient to remove the (weakly adsorbed) homopolymers.

The various structures that can be formed during laccase-catalyzed modification of PES surfaces have very different effects on the adhesion of model foulants such as proteins, polysaccharides and polyphenols (Chapter 5), and *Listeria monocytogenes* (Chapter 6). It was shown that surface hydrophilicity is not the only factor responsible for (prevention of) adhesion.

The average flux of the base membrane was never reduced more than 20% (mostly below 10%) in case of modification with 4-hydroxybenzoic acid, and less than 9% for gallic acid,

while the mechanical properties and thermal stability of the membrane were not adversely affected (Chapter 4).

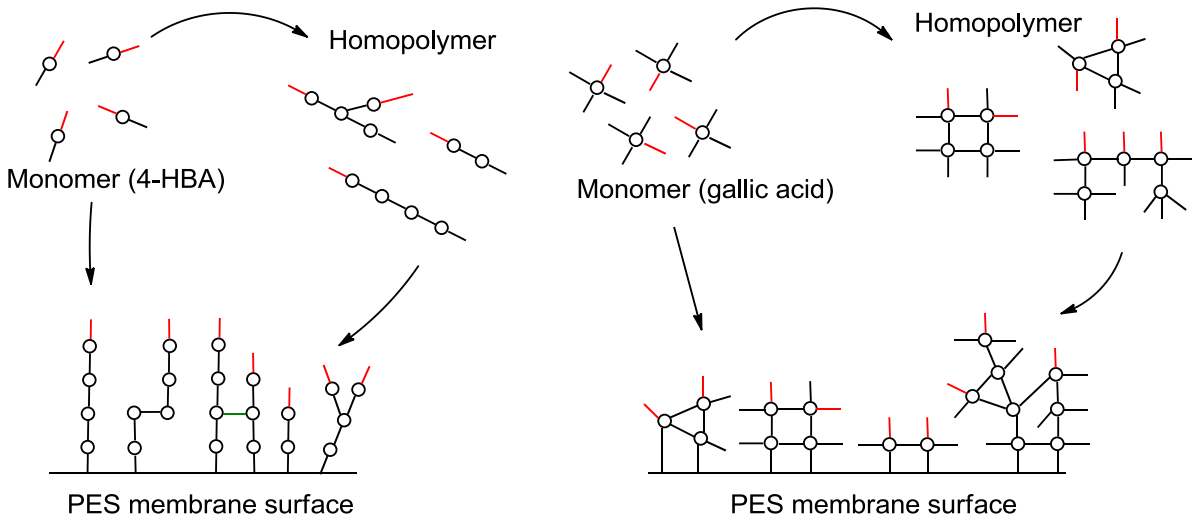


Figure 3. Schematic representation of modification layers formed by 4-hydroxybenzoic acid (4-HBA) and gallic acid. Black line: OH group or ether linkage; green line: C-C bond; red line: COOH group.

In conclusion, the laccase-catalyzed modification of PES membranes (surfaces) is a method that can be applied to flexibly create surface structures that can be used to effectively prevent (or possibly facilitate as will be discussed later) attachment of various components to the modified surface. The method can be carried out under very mild reaction conditions, and when applied to PES membranes the resulting membranes retain a high flux. Modified PES membranes possess mechanical properties and thermal resistances that are similar or superior to those of the base membrane, as was illustrated in Chapter 4. All these aspects make the presented method an interesting alternative for currently used modification methods, as was illustrated in Chapter 2.

2. Practical Aspects

In this section, practical aspects needed to bring the developed method to large-scale application are discussed, starting with the first results on hollow fibre membranes, which are presented together with an assessment of the costs involved in the method, and a list of other aspects.

The first results for hollow fibres were obtained with single high flush fibre modules which were kindly provided by Pentair X-flow (0.79 mm inner diameter and pore size that is

expected to be around 10 nanometers). The modification procedure was as follows. 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 ± 1 °C) was rinsed through the fibre by gravity (4 cm height difference between both ends of the module; outlet flow ~1 $ml \cdot min^{-1}$). Next, 20 ml of fresh well-mixed 4-hydroxybenzoic acid and laccase (28.8 mM and 0.5 $U \cdot ml^{-1}$, respectively) were rinsed through the module also by gravity. Freshly mixed reactants replaced the previous mixture every 15 min during a total modification time of 2 h. The modified membrane was cleaned by forward and backward washing with Milli-Q water (as described in Chapter 4) at 4 bar and the flux was measured. Next, 1 $g \cdot l^{-1}$ BSA was pressurized at 4 bar (pH 7, 0.1 M sodium acetate buffer, 24 ± 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 blank membranes (unmodified membranes).

Table 1. Specification and performance of the blank and modified high flush surface hollow single fibre PES lab modules.

	Blank module no.1	**Blank module no.2**	**Modified module no.1**	**Modified module no.2**
Length (cm)	23.7	24	23.6	24
Area (cm^2)	5.88	5.96	5.86	5.96
Flux ($m^3 \cdot m^{-2} \cdot h^{-1}$)	0.3 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
Flux after modification ($m^3 \cdot m^{-2} \cdot h^{-1}$)			0.072 ± 0.001	0.1 ± 0.001
Flux during BSA filtration ($m^3 \cdot m^{-2} \cdot h^{-1}$)	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
Flux after BSA adsorption ($m^3 \cdot m^{-2} \cdot h^{-1}$)	0.04 ± 0.001	0.04 ± 0.001	0.043 ±0.001	0.043 ± 0.001

Table 1 shows an overview of the results. The flux of modified membranes is typically 20-30% of that of the blank membrane, probably due to the presence of the relatively large modification layers compared to the pore size (in previous chapters larger pores were used: 0.2 µm). Upon exposure to BSA, the flux of the blank membrane was reduced to

approximately 12% of its original value due to pore blocking by BSA. For the modified membrane, slightly higher fluxes were found; obviously the relative flux decrease due to BSA was much less for these membranes. The most striking difference between the membranes is in BSA rejection, which was around 50% for modified membranes where it was almost 100% for the blank (unmodified) PES membrane. For unmodified membranes, accumulation and aggregation of protein will take place, which reduces the pore size and increases rejection. The lower rejection of the modified membranes is expected to be a result of reduced protein adsorption on/into the membrane pores (schematically illustrated in Figure 4), which allows passage of the protein through the membrane without sticking onto the pore mouth or surface. In this it is noteworthy that protein rejection of the membrane *surfaces* is different from protein rejection by the membrane itself: a perfectly protein-repellent surface will correlate with a completely non-rejecting membrane if the pore size allows unhindered passage of the protein. In this case, although the pore size of around 10 nm may be reduced (as reflected in flux reduction) upon modification by growth of a brush-like layer of around 3 nm on each side, the size of the modified pore may still be sufficient for passage of an individual protein molecule (BSA size is around 3-4 nm) [1]. This phenomenon opens an opportunity for size-based separation of the same protein type.

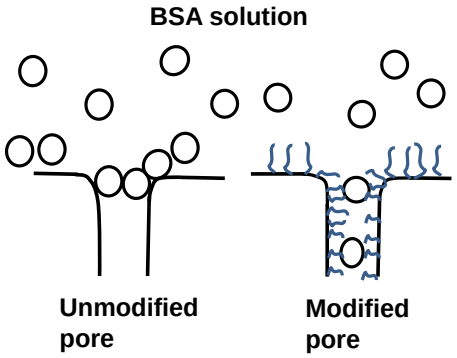


Figure 4. Schematic representation of the effect of modification of PES membrane pores on rejection of proteins.

For industrial application, an assessment of the costs involved in the enzyme-catalyzed modification method is needed. Both the enzyme and the used substrates are available commercially and not expensive, and it is possible to recycle the enzyme, substrate, and buffer. Table 2 shows an indication for the price of modification of one square meter of membrane with 4-hydroxybenzoic acid (28.8 mM modifier, 0.5 $U \cdot ml^{-1}$ enzyme) according to prices in the Sigma-Aldrich catalogue 2011. Based on the prices of the largest packages

available in the catalogue, the cost of modification chemicals is 120-130 euro per m^2. However, for larger scale production this price can likely be significantly reduced to a calculated cost of approximately 10 euro·m^{-2} when applied on industrial scale.

From the list it is clear that the enzyme cost is prominent, and when produced at large scale this cost will be reduced considerably too. For a commercial enzyme that is *e.g.* used in washing powder, a typical price would be 100 euro per kg of crude enzyme. The activity of such enzyme will be less as the one we used in our experiments, but even if the enzyme activity is a factor of 10 lower, the price for modification would still reduce with approximately a factor of 50. Alternatively the reaction could also be carried out at lower enzyme concentration, although this would require longer reaction times, which typically increases the overall costs**.**

Table 2. Price indication for modification of one m^2 of poly(ethersulfone) membrane.

Material	**Sigma-Aldrich 2011**	**Used amount/0.002 m^2 membrane.**	**Cost/m^2 membrane (euro/m^2)**
Sodium acetate	43.1 euro/1 kg	0.11 g	2.4
Acetic acid	13.5 euro/1 l	0.04 ml	0.3
Enzyme	38.6 euro/1 g	6 mg	115.4
4-hydroxybenzoic acid	48 euro/1 kg	0.2 g	4.8
		Total cost/m^2	122
On industrial scale the price can be significantly reduced, leading to a price per m^2 of:			10

*The costs related to oxygen needed for modification are negligible.

Besides the direct costs illustrated in Table 2, this enzyme-catalyzed modification has many positive aspects. Just 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 fibre modules, which is an important step for large-scale application. The method is reproducible as illustrated in the grafting yield and performance shown in chapter 4. 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 lead to reduced replacement costs.

3. Versatility and Future Developments

The enzymatic modification method can be taken one step further and applied to other substrates and surfaces. In the previous chapters, only one monomer has been used simultaneously, but obviously the technology is not limited to this. Using different substrates in consecutive (or simultaneous) reactions would allow multi-functionalization. For example, ferulic and 4-hydroxybenzoic acid can be used in combination, to see whether it is possible to make use of the anti-biofouling properties of ferulic acid and the anti-protein fouling properties of 4-hydroxybenzoic acid. Also, further functionalization of the modification layer can be obtained by using substrates that can subsequently react with other components, *i.e.*, with phenolic amines [2] as illustrated in Figure 5.

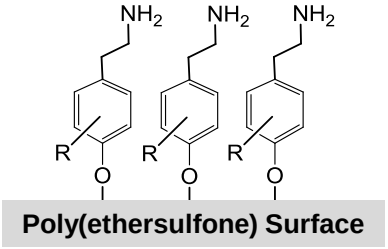


Figure 5. Proposed schematic representation of attachment of phenolic amines to the poly(ethersulfone) surface .

Besides PES, we found that other poly(aryl) surfaces such as poly(ether ether ketone) (PEEK, Victrex$^{@}$PEEK^{TM} Film Technology, 1000-150G, 150 micron thickness, see Figure 6) can be modified using 4-hydroxybenzoic acid, gallic acid, or ferulic acid via the same modification procedure.

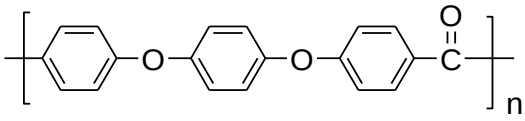


Figure 6. The structure of poly(ether ether ketone) (PEEK).

Color formation took place and the static water contact angle decreased, which indicates coupling of the phenolic acids to the polymer. For example, the contact angle of blank PEEK surface: 99.2 ± 4.3 (glossy side), 84.8 ± 1.5 (matte side) was measured. After modification using 4-hydroxybenzoic acid (28.8 mM), the contact angle becomes 57.9 ± 1.2 (glossy side), 51.1 ± 1.6 (matte side); after modification using gallic acid (4.8 mM) it becomes 60.3 ± 1.8 (glossy side), 58.8 ± 0.6 (matte side), and after modification using ferulic acid (4.8 mM) it

becomes 51.1 ± 1.0 (glossy side), 48.7 ± 3.2 (matte side). The previously mentioned results were obtained with the following modification conditions: 24 h modification time, 0.5 $U.ml^{-1}$ enzyme, 24 ±1 °C, and pH 5 (0.1 M sodium acetate buffer). Clearly, the enzyme-catalyzed modification method can be used for other poly(aryl) surfaces, but to what extent needs to be further investigated.

From this section, it is clear that laccase-catalyzed modification is a versatile method that can be used for other substrates, facilitates further functionalization, and can be used for other poly(aryl) surfaces. This all indicates that the method has the potential to be widely used in various fields of surface science and technology.

In summary, for the two applications targeted in this thesis, food and water treatment, the modified membranes seem to be well suited, and can still be further developed. Using a natural non-toxic compound (substrate) as modifier may show great benefits regarding safety issues for application in the food industry. Besides, it is expected that these modified membranes will be interesting for other applications, such as proton exchange membranes in fuel cells. Adding charged groups, such as carboxylic acids, to poly(arylsulfone) membranes will increase their proton conductivity (while keeping good water permeability), which makes these membranes an interesting alternative for the costly perfluorosulfonate membranes currently used, especially for application at high temperatures [3].

Reference

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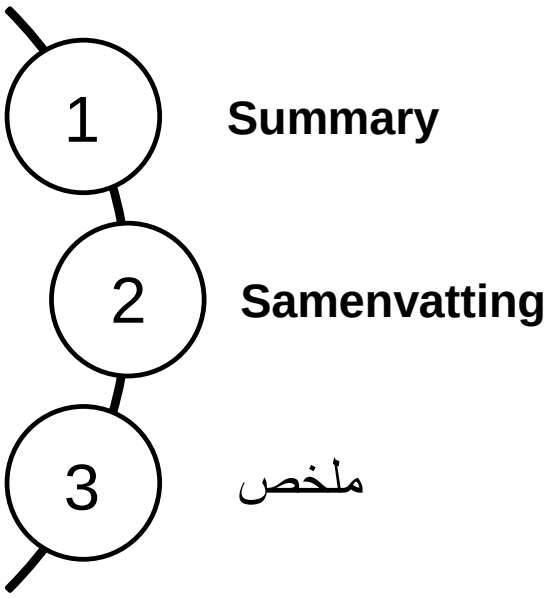
Summaries



Summary

Poly(ethersulfone) (PES) is the thermoplastic material of choice for the manufacture of ultrafiltration and microfiltration membranes, due to its structural and chemical stability. Unfortunately, the separation performance of PES membranes often deteriorates because of membrane fouling, which is attributed to the intrinsic hydrophobic character of this material. Therefore, introduction of different polar functional groups to the PES membrane surfaces through *e.g.* blending, coating, and radiation induced-grafting has been reported in literature. Although successful to some extent, these methods only offer random control over the resulting surface structure and may be environmentally adverse.

This study presents enzyme-initiated grafting of PES membranes as the first successful example of an environmentally friendly modification of PES membranes. Various phenolic acids, such as 4-hydroxybenzoic acid and gallic acid (3,4,5-trihydroxybenzoic acid), were coupled to the membrane in aqueous medium at room temperature using laccase from *Trametes versicolor* as catalyst. This enzyme is able to oxidize phenolic compounds to their corresponding free radicals that are subsequently grafted onto PES membranes, introducing polar groups (OH, COOH) on the membrane surface.

This thesis consists of seven chapters. **Chapter One** contains three sections: the first section concerns membrane separation processes and its drawbacks, with emphasis on fouling by proteins and microbial cells (biofouling). Also membrane materials are briefly presented in this section. The second section presents the enzyme laccase and the progress in its application from delignification to surface modification. Finally, the aim and the outline of the thesis are highlighted in the third section.

In **Chapter Two**, (surface) modification methods for poly(arylsulfone) [*i.e.*, polysulfone and poly(ethersulfone)] membranes are reviewed. All modification methods are compared on various aspects such as flux after modification, simplicity, reproducibility, environmental aspects, and cost effectiveness. In this review, enzyme-catalyzed modification is introduced as an environmentally benign alternative for other methods.

The principle and mechanism of the enzyme-catalyzed modification method is presented in **Chapter Three**. The modified membranes are studied using different analytical techniques (XPS, IRRAS, NMR). Reactive radicals produced by the action of laccase are grafted onto the surface of PES membranes by formation of a covalent C-O linkage as was proven by spin density calculations and IRRAS. Also, because of the presence of many phenolic radicals in

the reaction medium, extra monomers can be oxidatively grafted to the firstly attached monomer, with concurrent loss of both hydrogen atoms and carbon dioxide to form oligomers or polymers. At the same time, these extra monomers can also be oxidatively grafted to other monomers or pre-formed oligomers to form homopolymers that can be partially adsorbed to the membrane surface, which is especially relevant for fast-reacting phenolic acids such as gallic acid. Water is the only by-product in this enzymatic reaction. The flux of grafted membranes is not significantly influenced by the modification.

The performance of laccase-catalyzed modified PES membranes using 4-hydroxybenzoic acid and gallic acid as monomers/modifiers is evaluated in **Chapter Four**. Modified membranes show considerably reduced bovine serum albumin (BSA) adsorption. For 4-hydroxybenzoic acid, longer modification times and higher substrate concentrations lead to better protein repellence. Reactions with gallic acid are faster but less effective in terms of protein repellence. The difference in the number of hydroxyl groups per molecule between 4-hydroxybenzoic acid and gallic acid affects the structure of the modification layer and consequently the surface behavior against BSA. Attachment of 4-hydroxybenzoic acid, which has only one reactive hydroxyl group, leads mostly to linear structures (possibly with branches and/or collapsing especially at long modification times), which swell and extend in water and give rise to entropic repulsion (entropic brush formation). The three hydroxyl groups of gallic acid likely induce growth in three dimensions and network formation (*i.e.*, cross-linking and/or collapsing especially at high concentration and/or long modification times), which is less effective in protein repellence. Neither the clean water flux nor the thermal and mechanical properties of the modified membranes were significantly influenced. The latter is attributed to the low amount of grafted material (average 1% grafted material, increasing to around 2.5% at both long modification time and high phenolic acid concentrations). In addition, the modification layer is quite resistant against treatment at low and high pH.

Chapter Five explores the ability of enzymatically modified PES surfaces to repel foulants. BSA, dextrin, and tannin were used as model components for protein, polysaccharide, and polyphenol foulants. The effect of surface modification on the reversibly and irreversibly adsorbed amounts of foulants, in addition to the rate of adsorption, is studied using reflectometry. The obtained results show that at short modification times, the adsorption of the three foulants is reduced. However, at longer modification times, the adsorption increases again for both modifiers (4-hydroxybenzoic acid and gallic acid). The contact angle is reduced at short modification times but stays at the same low values at longer modification

times, which indicates that surface hydrophilicity is not the only determining factor for the measured differences. AFM imaging of the gallic acid-grafted PES surface as obtained using low phenolic concentration shows small islands at very short modification times (around 7 min), which then grow together and become denser and rougher upon longer modification times (>20 min). Such a compact layer apparently is less effective in foulant repellence as the more brush-like structure expected for 4-hydroxybenzoic acid. Apparently, formation of an effective foulant-repellent modification layer depends both on the structure of the used substrate (*e.g.* phenolic acid) and the modification conditions. Via each of these, the extent of (fast) surface grafting and (slower) intra-layer cross-linking, and/or branching, and/or collapse of the polymer chains can be controlled.

Chapter Six evaluates the ability of the modified model PES surfaces to resist both attachment and biofilm growth of the pathogenic bacterium *Listeria monocytogenes* under static and dynamic conditions. 4-Hydroxybenzoic acid, gallic acid, and ferulic acid were used as modifiers; the EGD-e and LR-991 strains of *L. monocytogenes* were used as the standard and the well-known biofilm former strains, respectively. The standard plate count method was used for initial evaluation, and fluorescence microscopy was used to illustrate the results. The surfaces modified with the three tested compounds repelled *Listeria* adhesion up to 70% (static conditions) or 95% (dynamic conditions). Biofilm growth was inhibited up to about 70%, depending on the temperature (presence/absence of flagella), the modifier and the modification time. It is expected that further optimization will lead to better results.

Chapter Seven consists of three sections. The first section is a general discussion and an overview of the enzyme-catalyzed modification method of poly(ethersulfone) membranes and surfaces. An outlook for industrial scale and future developments are presented in the second and third section. It is shown that the costs for the enzyme-catalyzed surface modification method on industrial scale are reasonable and the proposed method can also be used to modify other poly(aryl) surfaces.

In conclusion, PES membranes can be modified with phenolic acids using laccase in water at ambient temperature. The enzyme-catalyzed modification method shows a remarkable flexibility, and allows careful tuning of the membrane properties in such a way that membrane fouling can be suppressed. Besides, the modification method does not influence the bulk properties of the membrane adversely, which makes this modification method an interesting eco-friendly alternative to currently used methods.

Samenvatting

Poly(ethersulfon) (PES) is een thermoplastisch materiaal dat vanwege zijn grote structurele en chemische stabiliteit veel gebruikt wordt voor de fabricage van membranen voor ultrafiltratie en microfiltratie. Helaas gaat het scheidend vermogen van PES membranen vaak snel achteruit vanwege vervuiling, die wordt toegeschreven aan het intrinsiek hydrofobe karakter van dit materiaal. Daarom wordt bijvoorbeeld gebruikt gemaakt van menging (blending), of bedekking (coating) met andere polymeren en covalente modificatie (grafting) met behulp van ioniserende straling. Hoewel er successen behaald zijn met deze methoden, hebben zij als nadelen dat ze beperkte controle over de oppervlaktestructuur geven en milieuonvriendelijk zijn.

In dit onderzoek wordt enzymatisch modificatie van PES membranen gepresenteerd als eerste succesvol voorbeeld van een milieuvriendelijke modificatie methode voor deze oppervlakken. Diverse fenolische zuren zoals 4-hydroxybenzoëzuur en galluszuur (3,4,5-trihydroxybenzoëzuur) worden gekoppeld aan PES in waterig milieu bij kamertemperatuur met behulp van laccase uit *Trametes versicolor* als biokatalysator. Dit enzym is in staat om fenolen te oxideren tot de overeenkomstige vrije radicalen die op hun beurt covalent binden aan PES membranen. Hierdoor worden er polaire groepen (OH, COOH) geïntroduceerd op het membraanoppervlak.

Het proefschrift bestaat uit zeven hoofdstukken. **Hoofdstuk Een** bestaat uit drie delen. Het eerste deel bespreekt scheidingsprocessen met membranen en de nadelen daarvan, met nadruk op vervuiling door eiwitten en microbiële cellen (‘biofouling’). Ook membraanmaterialen komen kort aan de orde in dit deel. In het tweede gedeelte wordt het enzym laccase voor het voetlicht gebracht, inclusief de vorderingen die er gemaakt zijn in de toepassingen van laccase, van delignificatie tot en met oppervlaktemodificatie. Tot slot worden het doel van het onderzoek en de indeling van het proefschrift gepresenteerd.

In **Hoofdstuk Twee** worden de bekende (oppervlakte-) modificatiemethoden voor polyarylsulfonen [polysulfon en poly(ethersulfon)] oppervlakken besproken. De methoden worden vergeleken op basis van diverse aspecten zoals flux na modificatie, eenvoud van uitvoering, reproduceerbaarheid, milieuvriendelijkheid en kosten. In dit overzicht wordt enzymgekatalyseerde modificatie geïntroduceerd als milieuvriendelijk alternatief voor andere methoden.

Het principe en het mechanisme van de enzymgekatalyseerde modificatie van PES is het onderwerp van **Hoofdstuk Drie**. De gemodificeerde membranen zijn bestudeerd met verschillende analytische technieken (XPS, IRRAS, NMR). Uit IRRAS en spindichtheidsberekeningen bleek dat de door het laccase geproduceerde reactieve radicalen zijn gekoppeld aan het oppervlak van PES membranen d.m.v. een covalente C-O binding. Vervolgens kunnen er additionele fenolzuren oxidatief gekoppeld worden aan het oorspronkelijke adduct, onder gelijktijdige afsplitsing van twee waterstofatomen en CO_2, waarbij oligomeren of polymeren ontstaan. Gelijker tijd kunnen de monomeren ook oxidatief gekoppeld worden aan andere monomeren of oligomeren in oplossing, waarbij homopolymeren worden gevormd die aan het membraanoppervlak kunnen adsorberen. Dit is met name relevant voor snel reagerende fenolzuren zoals galluszuur. Water is het enige bijproduct van deze enzymatische reactie. De flux door de gemodificeerde membranen is niet significant veranderd door de koppelingsreactie.

De prestaties van de PES membranen die m.b.v. laccase gemodificeerd zijn met 4-hydroxybenzoëzuur en galluszuur worden geëvalueerd in **Hoofdstuk Vier**. De gemodificeerde membranen vertonen aanzienlijk minder adsorptie van runder serum albumine (BSA) dan de ongemodificeerde membranen. Bij 4-hydroxybenzoëzuur leiden langere modificatietijden en hogere substraatconcentraties tot betere eiwitafstoting. De reacties met galluszuur zijn sneller maar minder effectief in termen van eiwitafstoting. Het verschillende aantal hydroxylgroepen in 4-hydroxybenzoëzuur en galluszuur heeft gevolgen voor de structuur van de modificatielaag en daardoor voor het adsorptiegedrag van het oppervlak m.b.t. BSA. De structuur van oligomeren en polymeren van 4-hydroxybenzoëzuur, dat slechts één hydroxylgroep heeft, is vooral lineair, maar de vorming van vertakkingen en omgeklapte structuren is zeker mogelijk, met name bij langere modificatietijden. De lineaire structuren zwellen en strekken zich uit in water en leiden tot entropische afstoting (vorming van ‘entropische borstels’). De drie hydroxylgroepen van galluszuur daarentegen zullen eerder driedimensionale netwerken geven, waarbij crosslinking en inklinken van structuren vooral zullen voorkomen bij hogere concentraties en/of langere modificatietijden, waardoor deze structuren minder effectief zijn in eiwitafstoting. In alle gevallen heeft de modificatie nauwelijks effect op de schoon-water-flux van het membraan en de thermische en mechanische eigenschappen. Dit komt doordat er maar weinig materiaal op het PES membraan vastgehecht wordt: gemiddeld 1%, oplopend tot 2.5% bij lange incubatietijd met hoge fenolconcentraties. Bovendien is de modificatielaag redelijk goed bestand tegen behandeling met media van hoge en lage pH.

In **Hoofdstuk Vijf** wordt onderzocht hoe goed de enzymatisch gemodificeerde PES oppervlakken in staat zijn om adsorptie van vervuilende stoffen tegen te gaan. BSA, dextrine en tannine zijn gebruikt als voorbeelden voor eiwit, polysacharide en polyfenol vervuiling. Het effect van oppervlaktemodificatie op de reversibel en irreversibel gebonden hoeveelheid foulant, als ook de snelheid waarmee dit plaatsvindt, is onderzocht m.b.v. reflectometrie. De verkregen resultaten tonen aan dat de adsorptie van de drie modelstoffen verminderd wordt bij korte modificatietijden; echter, bij langere modificatietijden stijgt de adsorptie weer, voor zowel 4-hydroxybenzoëzuur als galluszuur als enzymsubstraat. De watercontacthoek is lager bij korte modificatietijd en blijft op hetzelfde lage niveau, ook bij langere modificatie, hetgeen aantoont dat hydrofiliciteit niet de enige factor van belang is voor adsorptie van macromoleculen. AFM imaging van PES oppervlakken gemodificeerd met lage concentraties galluszuur toont kleine eilandjes bij korte modificatietijd (ongeveer 7 minuten), die vervolgens aan elkaar groeien, dichter en ruwer worden bij langere tijd (> 20 minuten). Zo'n compacte laag is kennelijk minder effectief in de afstoting van vervuilende stoffen dan de meer borstelachtige structuren die verwacht worden bij 4-hydroxybenzoëzuur. Blijkbaar hangt de vorming van een effectieve foulant-afstotende laag van fenolzuren af van zowel de structuur van het gebruikte substraat (fenolzuur) als de modificatiecondities. Door variatie van beiden is volledige controle mogelijk over de mate van (snelle) oppervlaktemodificatie en (langzame) inwendige crosslinking en/of vertakking en/of inklappen van de polymeerketens.

Hoofdstuk Zes is gewijd aan het effect van enzymatische PES modificatie op het verhinderen van adhesie en biofilm vorming van de pathogene bacterie *Listeria monocytogenes*, zowel onder statische als dynamische condities. 4-Hydroxybenzoëzuur, galluszuur en ferulazuur zijn gebruikt als enzymsubstraten. Van *L. monocytogenes* is zowel de veelgebruikte EGD-e stam genomen als de LR-991 stam, die berucht is vanwege biofilm vorming. Voor initiële evaluatie is de plaat tel methode gebruikt; de resultaten zijn geïllustreerd m.b.v. fluorescentiemicroscopie. De oppervlakken die gemodificeerd zijn met de drie geteste verbindingen verminderen *Listeria* aanhechting tot maximaal 70% (statische condities) resp. 95% (dynamische condities). Biofilm vorming wordt voor maximaal 70% geremd, afhankelijk van de temperatuur (aan/afwezigheid van flagella), het gebruikte enzymsubstraat en de modificatietijd. Verdere optimalisatie zal leiden tot betere resultaten.

Hoofdstuk Zeven is verdeeld in drie secties. Het eerste deel bestaat uit een algemeen overzicht en discussie betreffende de enzymgekatalyseerde modificatiemethode van poly(ethersulfon) membranen en oppervlakken. Onze visie op industriële opschaling en toekomstige ontwikkelingen wordt gepresenteerd in het tweede en derde deel, respectievelijk.

De kosten van de enzymgekatalyseerde modificatiemethode lijken mee te vallen op industriële schaal, en verder kunnen ook andere poly(aryl) oppervlakken behandeld worden.

Concluderend, PES membranen kunnen gemodificeerd worden met fenolzuren m.b.v. laccase in water bij kamertemperatuur. De enzymgekatalyseerde modificatiemethode vertoont een verrassende flexibiliteit, waardoor membraaneigenschappen kunnen worden afgestemd op verminderde membraanvervuiling. Bovendien beïnvloedt de modificatiemethode de basiseigenschappen van het membraanmateriaal niet negatief, zodat het een interessant milieuvriendelijk alternatief is voor de bestaande methoden.

يقوم **الفصل السادس** بتقييم قدرة الأسطح النموذجية المعدَّلة للبولي (إيثر سلفون) على مقاومة ارتباط بكتيريا الليسترية المستوحدة الممرضة وتكدسها في شكل غلاف حيوي جرثومي في ظل الظروف الثابتة والمتغيرة. تم استخدام حمض 4-هيدروكسي بنزويك وحمض الغال وحمض الفيروليك كمواد معدِّلة، بينما تم استخدام فصائل EGD-e وLR-991 من بكتيريا الليسترية المستوحدة كنماذج تقليدية ومنتشرة للفصائل المكونة للغلاف الحيوي الجرثومي، على التوالي. تم استخدام طريقة التعداد التقليدية في التقييم المبدئي، كما استُخدم الفحص المجهري التألقي في عرض النتائج. ثُبِّط التصاق الليسترية في الأسطح التي تم تعديل تركيبها بالمركبات الثلاثة المختبرة، بنسبة تصل إلى 70% (في الظروف الثابتة\محلول ثابت) أو 95% (في الظروف المتغيرة\محلول جارٍ). تم تثبيط تكوين الغلاف الحيوي الجرثومي بنسبة 70%، الأمر الذي يعتمد على درجة الحرارة (وجود\غياب السياط في البكتيريا)، وعلى المادة المعدِّلة وفترة التعديل. ومن المتوقع أن إجراء المزيد من التحسينات على العملية سيؤدي إلى نتائج أفضل.

ينقسم **الفصل السابع** إلى ثلاثة أجزاء. يتضمن الجزء الأول مناقشةً واستعراضاً عاماً لطريقة التعديل المحفزّة بالإنزيم لأغشية وأسطح البولي (إيثر سلفون). أما في الجزئين الثاني والثالث، فيتم مناقشة إمكانية التطبيق على النطاق الصناعي والتحديثات التي يمكن إجراؤها مستقبلاً. ثبت أن تكلفة استخدام طريقة تعديل الأسطح المحفزّة بالإنزيم تعد معقولة على النطاق الصناعي، كما يمكن للطريقة المقترحة أن تُستخدم كذلك لتعديل أسطح أخرى للبولي (أريل).

وكنتيجة للدراسة، يمكن تعديل أغشية البولي (إيثر سلفون) بالأحماض الفينولية باستخدام إنزيم اللاكاز (Laccase) في الماء، وفي درجة حرارة البيئة المحيطة. توفر طريقة التعديل المحفزّة بالإنزيم مرونة ملحوظة، وتتيح الضبط الدقيق لخصائص الغشاء بشكل يسمح بالحد من تلوثه. بالإضافة إلى ذلك، لا تؤثر طريقة التعديل هذه على الخصائص الأساسية للغشاء بشكل سلبي، مما يجعلها بديلاً مثيراً للاهتمام وصديقاً للبيئة مقارنة بالطرق المستخدمة حالياً.

أمر ذو صلة بشكل خاص بالأحماض الفينولية سريعة التفاعل مثل حمض الغال. يعد الماء هو المنتج الثانوي الوحيد في هذا التفاعل الإنزيمي. ولا يتأثر تدفق الأغشية المطعمة بشكل كبير بعد التعديل.

تم تقييم أداء أغشية البولي (إيثر سلفون) المعدلة والمحفزة بإنزيم اللاكاز (Laccase) باستخدام حمض 4-هيدروكسي بنزويك وحمض الغال كمونومرات\مواد معدِّلة في **الفصل الرابع**. تظهر الأغشية المعدلة انخفاضاً كبيراً في امتزاز ألبومين المصل البقري (BSA). وبالنسبة لحمض 4-هيدروكسي بنزويك، فإن فترات التعديل الأطول والتركيزات الأعلى له تؤدي إلى تكوين طبقة معدلة قادرة على طرد البروتين بفاعلية. وتكون التفاعلات مع حمض الغال أسرع ولكنها تكون طبقة معدلة أقل فاعلية فيما يتعلق بطرد البروتين. يؤثر الاختلاف بين عدد المجموعات الهيدروكسيلية لكل جزيء بين حمض 4-هيدروكسي بنزويك وحمض الغال على تكوين الطبقة المعدلة وبالتالي السلوك السطحي في مقابل ألبومين المصل البقري. إن إضافة حمض 4-هيدروكسي بنزويك، والذي يحتوي على مجموعة هيدروكسيلية متفاعلة واحدة فقط، تؤدي في أغلب الأوقات إلى تكوين مركبات طولية (وربما مع وجود تفرّيعات و\أو تهدم وخاصة في فترات التعديل الطويلة)، والتي تتزايد وتمتد في الماء وتزيد من التنافر الإنتروبي (التكوين الإنتروبي الشبيه بالفرشاة). تقوم مجموعات الهيدروكسيل الثلاثة في حمض الغال على الأرجح بتحفيز النمو ثلاثي الأبعاد وتكوين شبكة جزيئية (أي تحفيز التشابك و\أو تهدم، خاصة في التركيزات المرتفعة و\أو فترات التعديل الطويلة)، مما يجعله أقل فعالية في طرد البروتينات. لم يكن هناك تأثير ملحوظ على تدفق المياه النظيفة ولا على الخصائص الحرارية والميكانيكية للأغشية المعدلة. يرجع عدم تأثر الخصائص الحرارية والميكانيكية إلى قلة كمية المادة المطعمة (1 % مادة مطعمة في المتوسط، وتزيد إلى حوالي 2.5 % في فترة التعديل الطويلة والتركيزات المرتفعة للحمض الفينولي). بالإضافة إلى ذلك، فإن الطبقة المعدَّلة تقاوم إلى حد كبير المعالجة في درجات الحمضية المنخفضة والمرتفعة.

يستكشف **الفصل الخامس** قدرة أسطح البولي (إيثر سلفون) المعدلة إنزيمياً على طرد الملوثات. تم استخدام الألبومين المصلي البقري (BSA) والدكسترين والتانين كنماذج لملوثات من نوع البروتينات وعديدات السكريات والبولي فينولات. تتم دراسة أثر تعديل التركيب السطحي على كميات الملوثات الممتزة بشكل دائم (غير استرجاعي) أو استرجاعي، بالإضافة إلى معدل الامتزاز، وذلك عن طريق مقياس الانعكاس. تُظهر النتائج التي تم الحصول عليها أنه في فترات التعديل القصيرة، ينخفض معدل امتزاز أنواع الملوثات الثلاثة. وبالرغم من ذلك، ففي فترات التعديل الطويلة، يزيد الامتزاز مجدداً لكلا المركبين المعدِّلين (حمض 4-هيدروكسي بنزويك وحمض الغال). تقل زاوية التماس في فترات التعديل الطويلة، إلا أنها تبقى بنفس قيمها المنخفضة في فترات التعديل الطويلة، مما يشير إلى أن خاصية السطح المحبة للماء ليست هي العامل المحدد الوحيد للاختلافات التي تم قياسها. إن التصوير بمجهر الطاقة الذرية (AFM) لسطح البولي (إيثر سلفون)- المعالج بحمض الغال والذي تم الحصول عليه باستخدام تركيزات فينولية منخفضة، يُظهر تكوينات تشبه جزراً صغيرة في فترات التعديل القصيرة (حوالي 7 دقائق)، تنمو تلك الجزر بعد ذلك وتندمج معاً وتصبح أكثر كثافة وخشونة مع فترات التعديل الطويلة (>20 دقيقة). من الواضح أن مثل هذه الطبقة المدمجة تكون أقل فعالية في طرد الملوثات من التركيب الأكثر شبهاً بالفرشاة والمتوقع مع استخدام حمض 4-هيدروكسي بنزويك. من الواضح، أن تكوين طبقة معَّدلة فعالة في طرد الملوثات، يعتمد على كل من تركيب المادة المعدِّلة (على سبيل المثال الحمض الفينولي) وظروف عملية التعديل. من خلال كلا منهما، يمكن التحكم في مدى تطعيم السطح (السريع) والتشابك بين-الطبقي (الأكثر بطئاً) و\أو التفرّع، و\أو تهدم سلاسل البوليمر.

ملخص

إن البولي (إيثر سلفون) هي إحدى المواد الثيرموبلاستيكية الأكثر شيوعاً في صناعة أغشية الترشيح فائق الدقة والترشيح الدقيق، وذلك بفضل استقرارها التكويني والكيميائي. ولسوء الحظ، فإن الأداء الفاصل لأغشية البولي (إيثر سلفون) غالباً ما يتدهور بسبب تلوث الغشاء، وهو الأمر الذي يُعزى إلى الخاصية المميزة لهذه المادة بكونها كارهة للماء. ولذلك، فقد تم إدخال مجموعات وظيفية قطبية مختلفة على أسطح أغشية البولي (إيثر سلفون) بواسطة عدة طرق، منها على سبيل المثال الدمج والتغليف و التطعيم المستحث بالإشعاع بحسب ما ورد في المنشورات العلمية. وعلى الرغم من كون هذه الطرق ناجحةً إلى حدٍ ما، إلا أن كل ما تقدمه هو تحكم عشوائي في تكوين السطح الناتج كما أنها قد تكون ضارة من الناحية البيئية.

تقدم هذه الدراسة التطعيم المحفز بالإنزيم لأغشية البولي (إيثر سلفون) باعتباره أول مثال ناجح على تعديل صديق للبيئة لأغشية البولي (إيثر سلفون). تم إقران العديد من الأحماض الفينولية، مثل حمض 4-هيدروكسي بنزويك وحمض الغال (3،4،5- حمض تريهيدروكسي بنزويك)، مع الغشاء في وسط مائي في درجة حرارة الغرفة وذلك باستخدام إنزيم اللاكاز (Laccase) المستخرج من فطر *Trametes versicolor* كمادة محفزة. حيث يتمكن هذا الإنزيم من أكسدة المركبات الفينولية إلى جذورها الحرة والتي يتم تطعيمها على أغشية البولي (إيثر سلفون)، وبالتالي إدخال مجموعات قطبية (الهيدروكسيل، الكربوكسيل) على سطح الغشاء.

وتتكون هذه الرسالة من سبعة فصول. يتضمن **الفصل الأول** ثلاثة أقسام: يتعلق القسم الأول بعملية الفصل بالأغشية وسلبياتها، مع التأكيد على التلوث الناجم عن امتزاز البروتينات والخلايا الميكروبية (التلوث الحيوي). كما يتم عرض مواد الأغشية بصورة موجزة في هذا القسم. ويعرض القسم الثاني إنزيم اللاكاز (Laccase) والتطور الذي يحدث في عملية استخدامه بدءاً من إزالة التخشب وحتى تعديل التركيب السطحي. وفي الختام، يتم تسليط الضوء على الهدف من الرسالة ومحتوياتها في القسم الثالث.

في **الفصل الثاني**، يتم استعراض طرق تعديل (التركيب السطحي) لأغشية البولي(أريل سلفون) [أي، البولي سلفون والبولي(إيثر سلفون)]. تتم مقارنة جميع طرق التعديل من العديد من الجوانب مثل مدى التدفق بعد التعديل والبساطة وقابلية إعادة الإنتاج والجوانب البيئية والتكلفة. وفي هذا الاستعراض، يتم تقديم عملية التعديل المحفزة بالإنزيم بوصفها بديلاً غير ضارٍ من الناحية البيئية للطرق الأخرى.

يتم عرض فكرة وآلية عمل طريقة التعديل المحَفَز بالإنزيم في **الفصل الثالث**. وتتم دراسة الأغشية المعدلة باستخدام أساليب تحليلية مختلفة (التحليل الطيفي الضوئي الإليكتروني باستخدام الأشعة السينية (XPS)، التحليل الطيفي لامتصاص الأشعة تحت الحمراء (IRRAS)، الرنين النووي المغناطيسي (NMR)). يتم تطعيم الجذور المتفاعلة الناتجة عن نشاط إنزيم اللاكاز (Laccase) فوق سطح أغشية البولي (إيثر سلفون) من خلال تكوين ترابط تساهمي (تكوين لمجموعة كربونيل)، حيث تم إثبات ذلك من خلال حسابات الكثافة المغزلية والتحليل الطيفي لامتصاص الأشعة تحت الحمراء. وبالإضافة إلى ذلك ونتيجة لوجود العديد من الجذور الفينولية في وسط التفاعل، فيمكن تطعيم مونومرات إضافية عن طريق الأكسدة على المونومر المضاف في البداية، مع الفقدان المتزامن لكلٍ من ذرات الهيدروجين وثاني أكسيد الكربون لتكوين أوليجومرات أو بوليمرات. وفي نفس الوقت، فقد يتم تطعيم هذه المونومرات الإضافية عن طريق الأكسدة على مونومرات أخرى خاصة بأوليجومرات متكونة مسبقاً لتكوين هوموبوليمرات يمكن امتزازها جزئياً إلى سطح الغشاء، وهو

Publication List

Grafting of Poly(arylsulfone) and a Process of Grafted Poly(arylsulfone)
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Modification Methods for Poly(arylsulfone) Membranes: A Mini-Review Focusing on Surface Modification
Norhan Nady, Maurice Franssen, Han Zuilhof, Mohamed Mohy Eldin, Remko Boom, and Karin Schroën, Desalination 275 (2011) 1-9.

Mild and Highly Flexible Enzyme-Catalyzed Modification of Poly(ethersulfone) Membranes
Norhan Nady, Karin Schroën, Maurice Franssen, Barend van Lagen, Sukumaran Murali, Mohamed Mohy Eldin, Remko Boom, and Han Zuilhof, H., ACS Appl. Mater. Interfaces 3 (2011) 801-810.

Laccase-Catalyzed Modification of PES Membranes with 4-Hydroxybenzoic Acid and Gallic Acid
Norhan Nady, Karin Schroën, Maurice Franssen, Mohamed Mohy Eldin, Han Zuilhof, and Remko Boom, J. Membr. Sci. 394-395 (2012) 69-79.

Enzyme-Catalyzed Modification of PES Surfaces: Reduction in Adsorption of BSA, Dextrin and Tannin
Norhan Nady, Karin Schroën, Maurice Franssen, Remco Fokkink, Mohamed Mohy Eldin, Han Zuilhof, and Remko Boom, to be submitted.

Listeria monocytogenes Repellence by Enzyme-Catalyzed Modified PES Surfaces
Norhan Nady, Stijn van der Veen, Karin Schroën, Maurice Franssen, Mohamed Mohy Eldin, Tjakko Abee, Han Zuilhof, and Remko Boom, in preparation.

Overview of Completed Training Activities

Discipline Specific Activities

Courses

- Bioreactors Design and Operation, the Netherlands (VLAG, 2008)
- Nanoparticle Technology, the Netherlands (OSPT, 2009)
- NanoMemCourse EA3: Nano-Structured Materials and Membranes in the Food Industry, Italy. (ITM-CNR, 2010, Poster)

Conferences, Workshops, and Symposia

- 1st Conference on Contemporary Environmental Issues in Arid and Semi-Arid Regions, Egypt (Alexandria University, 2008)
- Workshop on your Factory and the Environment: Industrial Wastewater and Environmental Laws, Egypt (ATNMRI, 2009)
- Food Process Engineering Internal Symposium, the Netherlands (2009,Oral)
- NPS9: Process Technology at the Interface, the Netherlands (2009, Oral)
- Meeting of KNCV "Design & Synthesis", "Structure & Reactivity" and "Biomolecular Chemistry", the Netherlands (2010)
- BerlinFOOD2010, Germany (2010, Oral)
- Nano4water Workshop, Germany (2010)
- 13th Aachener Membrane Kolloquium, Germany (2010, Poster)
- Food Process Engineering Internal Symposium, the Netherlands (2010/11)
- International Congress on Membranes and Membrane Processes, the Netherlands (ICOM 2011, Oral)
- Desalination Workshop, the Netherlands (2011)
- NPS 11: From Plan to Plant, the Netherlands (2011, Oral)
- Netherlands MicroNanoConference '11, the Netherlands (2011, Oral)

General Courses

- Information Literacy, including Introduction EndNote (WGS, 2009)
- Project and Time Management (WGS, 2009)
- Techniques for Writing and Presenting a Scientific Paper (WGS, 2009)
- Philosophy and Ethics of Food Science & Technology (VLAG, 2010)
- English Course (Eurolinguist taleninstituut, 2011)

Optionals

- Preparation PhD Research Proposal (2008)
- Research Progress Meetings (2008-11)
- Food Process Engineering Group Meetings (2009-11)
- Organic Chemistry Group Meetings (2009-11)
- PhD-trip Food Process Engineering (USA, 2010)

This list has been approved by VLAG

Acknowledgements

I owe my deepest gratitude to my family for their continuous support during my life. Your continuous encouragement and advice were the inspiration needed to achieve this work. My darlings, you are in my life, I own you the entire world! To my family I dedicate this thesis.

I am indebted to my daily supervisors, Assoc. Prof. Karin Schroën and Assoc. Prof. Maurice Franssen. Dear Karin and Maurice, I cannot find enough suitable words to express my appreciation for the massive amount of time you dedicated in the form of guidance and assistance during these four years of continuous work. I appreciate your great efforts in explaining things clearly and simply, which enabled me to develop the subject and to lend words of encouragement. I feel motivated and encouraged every time I attend your meetings. Throughout my thesis-writing period, you provided advice, teaching, and lots of good ideas. I would have been lost without both of you. Without your encouragement and guidance this project would not have materialized. Also thanks to Prof. M.S. Mohy Eldin for his encouragement.

I would like to show my greatest appreciation to the thesis supervisors; Prof. Han Zuilhof and Prof. Remko Boom. Dear professors, I can't thank you enough for your valuable comments, ideas, and insight, which have been of great value to me and motivated this work.

My sincere thanks to Remco Fokkink and Barend van Lagen for the fruitful discussions and valuable contributions to this work. Also, thanks to Astrid Roosjen for coaching me on ellipsometry and reflectometry and to Stijn van der Veen for his support in the Food Microbiology Lab. Thanks are also expressed to Naglaa Roushdy, Nahala Taha, Mahmoud Darwish, Mohamed Fahmy, Marcel Giesbers, Tiny Franssen-Verheijen, Frank Claassen, Elbert van der Klift, Jacob Baggerman, and Xandra Bakker-de Haan for their instrumental assistance in both Egypt and the Netherlands.

I would like to thank all the members of the ShIFT project (FO-00-01) and its leader André Mepschen for critical discussions and encouragement during the past two years.

This thesis would not have been possible without the essential and gracious support of many individuals: Joyce Krauss, Jos Sewalt and Maurice Strubel; thanks a lot for your help. I would like to thank all my colleagues in both Egypt and the Netherlands who supported me during this work. I am deeply indebted to Hassan Sawalha and his wife Amal Samaaneh, Wasma Al-Husainy and Lina Abu Ghunmi. I am very grateful for your gracious support and encouragement. Thanks a lot.

Last but not least, I offer my regards and blessings to all of those who supported me in any respect during the completion of this thesis.

About the Author

Norhan Nady Ibrahim Mohamed Kotb was born in Cairo, Egypt on 16th of July 1977. After graduating from secondary school, she moved with her family to Alexandria. In June 2002, she obtained the BSc. degree in Chemical Engineering from Alexandria University (Overall grade: very good, graduation project: distinction). In September 2002, she started her MSc project in membrane fabrication with title "Novel Asymmetric Membranes Suitable for Ultrafiltration and Reverse Osmosis". In April 2003, she worked at Alexandria Shipyard as a Production/Chemical Engineer in the fiber glass workshop. In December 2004, she worked at Polymeric Material Research Department, Advanced Technology and New Material Research Institute (ATNMRI), Alexandria as a Research Assistant. Norhan defend her MSc. in May 2007 and raised her academic position to Assistant Researcher. In October 2007, she was accepted as PhD student "Sandwich System" in Wageningen University to start her research between Egypt (ATNMRI) and the Netherlands (Food Process Engineering Group - Laboratory of Organic Chemistry) in February 2008. The results of her research are presented in this thesis.

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The research described in this thesis was funded by ISPT.
The cover: Green global image has been printed with a permission from istockphoto.
Translation into Arabic: "Arabic Localizer" www.arabiclocalizer.com.

Printed by GVO printers & designers B.V./ Ponsen & Looijen, Ede.