ACS APPLIED MATERIALS & INTERFACES
Research Article
www.acsami.org

Functionalization of a Membrane Sublayer Using Reverse Filtration of Enzymes and Dopamine Coating

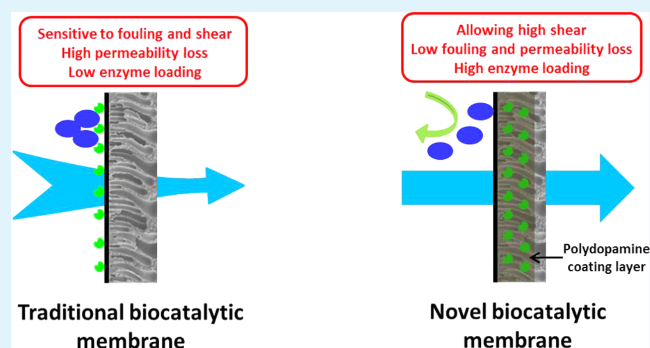
Jianquan Luo,*,† Anne S. Meyer,† R.V. Mateiu,‡ Dayanand Kalyani,† and Manuel Pinelo*,†

†Department of Chemical and Biochemical Engineering, Center for Bioprocess Engineering, Technical University of Denmark, Building 229, DK-2800 Kongens Lyngby, Denmark

‡Center for Electron Nanoscopy, Danchip, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark

Supporting Information

ABSTRACT: High permeability, high enzyme loading, and strong antifouling ability are the desired features for a biocatalytic membrane to be used in an enzymatic membrane reactor (EMR). To achieve these goals, the membrane sublayer was enriched with laccase by reverse filtration in this case, and the resulting enzyme-loaded sublayer was covered with a dopamine coating. After membrane reversal, the virgin membrane skin layer was facing the feed and the enzymes were entrapped by a polydopamine network in the membrane sublayer. Thus, the membrane sublayer was functionalized as a catalytically active layer. The effects of the original membrane properties (i.e., materials, pore size, and structure), enzyme type (i.e., laccase and alcohol dehydrogenase), and coating conditions (i.e., time and pH) on the resulting biocatalytic membrane permeability, enzyme loading, and activity were investigated. Using a RC10 kDa membrane with sponge-like sublayer to immobilize laccase with dopamine coating, the trade-off between permeability and enzyme loading was broken, and enzyme loading reached 44.5% without any permeability loss. After 85 days of storage and reuse 14 times, more than 80% of the immobilized laccase activity was retained for the membrane with a dopamine coating, while the relative activity was less than 40% without the coating. The resistance to high temperature and acidic/alkaline pH was also improved by the dopamine coating for the immobilized laccase. Moreover, this biocatalytic membrane could resist mild hydrodynamic cleaning (e.g., back-flushing), but the catalytic ability was reduced by chemical cleaning at extreme pH (e.g., 1.5 and 11.5). Since the immobilized enzyme is not directly facing the bulk of EMRs and the substrate can be specifically selected by the separation skin layer, this biocatalytic membrane is promising for cascade catalytic reactions.



KEYWORDS: *biocatalytic membrane, EMR, laccase, mussel-inspired coating, enzyme immobilization*

1. INTRODUCTION

Enzymatic biocatalysis is considered a green technology, but in many processes, robust recycling or immobilization of enzymes is required for maximizing the biocatalytic productivity, especially for production of low-medium value products.[1−3] Among the existing methods of enzyme utilization, immobilizing enzymes on membranes is advantageous as a level of product separation can be achieved simultaneously with the biocatalytic reaction(s) in enzyme membrane reactors (EMRs). In such "flow-through" reactors, as the enzyme is "docked" in/on the membrane, the reaction takes place when the substrates pass through the membrane pores.[4] EMRs have evolved as a promising technology in food processing, pharmaceuticals, biorefinery, and wastewater treatment due to prolonged enzyme activity, reuse ability, reduction in costs, the capacity to operate in continuous mode, easy operation, and scale-up to large systems.[5−8] Moreover, the continuous removal of product can shift the equilibrium of a reaction toward the product side and thus increase the productivity of the whole process, which is another remarkable advantage of EMRs.[3,7]

Immobilization of enzymes in or on membranes can be achieved via adsorption, covalent bonding, cross-linking, or entrapment, all of which have their own benefits and drawbacks. There are a number of examples of immobilization of laccase on membranes. Lante et al. performed immobilization of laccase using a normal crossflow filtration with a 3 kDa polyethersulphone (PES) ultrafiltration (UF) membrane,[9] resulting in adsorption of laccase to the membrane. Georgieva et al. reported that laccase could be covalently immobilized on a chromic acid-modified polypropylene (PP) membrane.[10] Chea et al. first fabricated a gelation layer on a ceramic membrane and then immobilized laccase on the gelation layer using covalent bonding.[11] In the described laccase immobilization

Received: October 22, 2014
Accepted: November 24, 2014
Published: November 24, 2014

 © 2014 American Chemical Society

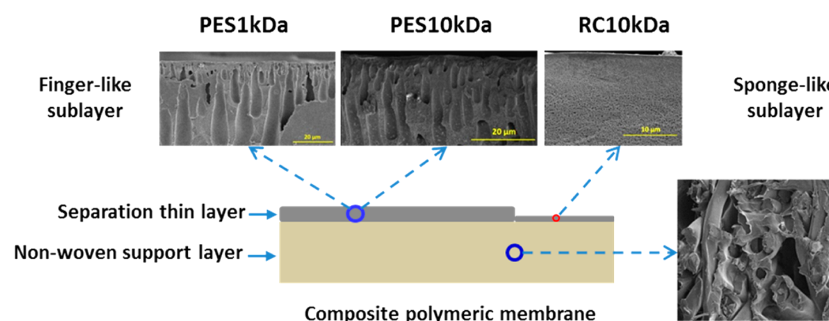


Figure 1. Structure and configuration of polymeric composite membranes.

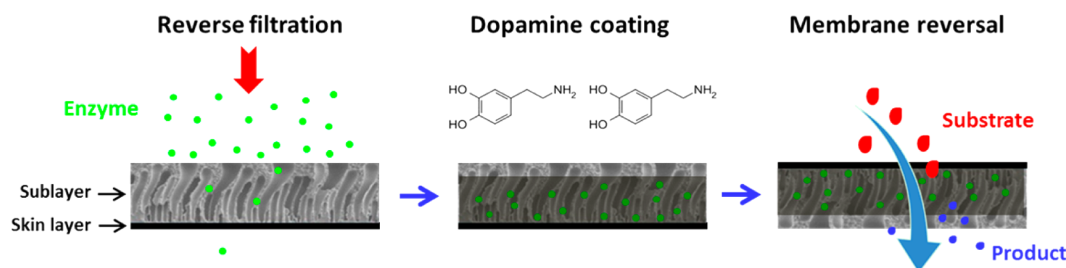


Figure 2. Schematic illustration of biocatalytic membrane prepared via fouling-induced enzyme immobilization and bioinspired coating. Enzyme immobilization was first carried out by reverse filtration of enzymes; then, the dopamine coating in/on the sublayer was conducted at pH 8.5, and finally, the membrane orientation was turned into the normal mode for the reaction.

strategies, enzyme loading was relatively low due to the limited attachment sites on the membrane surface.

In order to immobilize laccase on membranes with increased enzyme loadings, Hou et al. blended TiO_2 nanoparticles into PES to obtain a nanocomposite membrane for laccase immobilization.[12] Since the TiO_2 nanoparticles provided high surface areas for adsorption or covalent bonding of the laccase, the enzyme loading was greatly improved. However, due to the inherent limitations of the blending process, a large amount of the TiO_2 particles were embedded within the PES matrix and therefore were not accessible to the laccase during the immobilization process. Recently, Hou et al. fabricated a TiO_2 sol−gel coated polyvinylidene fluoride (PVDF) membrane for laccase immobilization, and the maximum enzyme loading by covalent bonding was more than 200 μg cm^{-2} as the TiO_2 particles were deliberately located on the surface of the membrane.[13] Nevertheless, for this novel and costly biocatalytic membrane using TiO_2 particles, renewing the enzyme bonding and reusing the membrane remain significant technical challenges to be addressed. In this regard, noncovalent enzyme immobilization is advantageous because the denatured enzymes can be removed by physical or chemical cleaning and then the membrane can be reused.

Regarding noncovalent enzyme immobilization, the enzyme could be immobilized within the porous spongy layer of a membrane by a simple pressure-driven filtration from shell to lumen using a hollow fiber membrane, where the enzymes cannot pass through the lumen side of the membrane and thus become entrapped in the membrane.[6,14] On the other hand, an asymmetric composite membrane could also be used and the enzyme was immobilized in/on the membrane sublayer by a reverse filtration (support layer facing feed). Since the immobilization mechanism of immobilizing an enzyme on the membrane sublayer by reverse filtration is similar to the mechanism of membrane fouling formation, such an enzyme immobilization process was termed "fouling-induced enzyme immobilization".[4,15] The described noncovalent immobilization strategies can achieve high enzyme loadings and allow easy renewability of the enzyme loading. However, in similarity to the above-mentioned methods of covalent laccase immobilization strategies described in the literature, there is a trade-off between final membrane permeability and enzyme loading for the resulting biocatalytic membranes.[12,14,15] Moreover, the enzyme-loaded membranes lose their original antifouling abilities because the membrane skin is modified by proteins (i.e., becomes less smooth and hydrophilic) or the sponge/support layer with low antifouling performance is facing the feed solution.[3]

To address the issues associated with enzyme immobilization, in the present report, we make an attempt to develop a novel enzyme immobilization strategy to build a biocatalytic membrane with high permeability and enzyme loading, as well as strong antifouling ability. For this purpose, fouling-induced enzyme immobilization[4,15] in different commercial composite membranes and dopamine coating are employed. Dopamine, an important hormone and neurotransmitter in the human body, can form a self-polymerized coating on various substrates under alkaline conditions and in an air atmosphere.[16] Polydopamine has also been called a "bio-glue", a property that is attributed to its adhesion ability and facile deposition process, which is currently used for membrane fabrication/modification[17−23] and enzyme immobilization.[24−28] The employed membranes consist of a nonwoven PP support layer and a separation thin layer made up of different materials and pore sizes. Here, as illustrated in Figure 1, the top, dense part of the separation layer is called the "skin layer" and the rest of the porous part, which is close to support layer, is termed the "sublayer". Figure 2 shows a schematic diagram of our novel strategy to immobilize enzymes in the membrane sublayer (the support layer was ignored due to its very large pore size). The hypothesis that supports this immobilization strategy is that the enzymes can be retained by the membrane skin layer and

accumulate in the sublayer by reverse filtration (support layer facing feed), and the dopamine coating will wrap the enzymes and "lock" them in the membrane pores. The polydopamine coating layer is thin and porous; thus, when the membrane orientation is returned to normal mode (skin layer facing feed), the original membrane permeability (that which was present before immobilization) can be maintained and the immobilized enzymes will not leak during the reaction. If the theorized strategy can be verified, the trade-off between permeability and enzyme loading in the biocatalytic membrane can be broken and the original antifouling ability of the membrane can be retained by this method. To the best of our knowledge, this is the first attempt to immobilize enzymes in a membrane with noncovalent attachment and dopamine coating. Laccase and alcohol dehydrogenase were used as examples for assessing this new strategy. The effects of membrane properties, coating time, and pH on the final membrane permeability, enzyme loading, and activity were examined and the stability of the biocatalytic membranes was also estimated. In addition, by comparing the results under different conditions (and with different enzymes), the mechanisms of this novel immobilization strategy are discussed.

2. MATERIALS AND METHODS

2.1. Materials. Laccase (EC 1.10.3.2, 60−70 kDa, 26.0 U mg^{-1}) from *Trametes versicolor* was purchased from Fluka. Alcohol dehydrogenase (EC 1.1.1.1, 141 kDa, >300 U mg^{-1}) from *Saccharomyces cerevisiae* (ADH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 0.55 kDa), dopamine hydrochloride (0.19 kDa), β-nicotinamide adenine dinucleotide reduced form (NADH, >97 wt %, 0.7 kDa), and formaldehyde (≥37%, 0.03 kDa) were purchased from Sigma-Aldrich. All the enzyme and substrate solutions were prepared using a 50 mM phosphate buffer except where stated otherwise. Dopamine hydrochloride was prepared freshly using a 10 mM Tris-HCl buffer (pH 8.5) or 50 mM phosphate buffer (pH 5.2). Three commercial polymeric composite membranes, NP010 (Microdyn-Nadir, PES, 1 kDa), GR81PP (Alfa Laval, PES, 10 kDa), and RC70PP (Alfa Laval, regenerated cellulose (RC), 10 kDa), were used in this work. On the basis of the membrane materials and pore size (i.e., molecular weight cutoff), NP010, GR81PP, and RC70PP are called PES1 kDa, PES10 kDa, and RC10 kDa in this paper, respectively.

2.2. Preparation of Biocatalytic Membranes. The enzyme immobilization, dopamine coating, and activity assay of immobilized enzymes were performed in a stirred cell (Amicon 8050, Millipore, USA). Descriptions of the equipment and procedure used can be found in our previous work.[4,15] The membranes were first soaked in 50% ethanol solution for 5 min and then washed with deionized water. After that, they were placed in the stirred cell in "sandwich" mode[15] (own support layer facing feed with an extra PP support beneath the skin layer). Afterward, the membranes were cleaned using a NaOH solution (0.1% for PES membranes and pH 10 for RC membranes) at 1 bar for 1 h, and then, the permeability was measured using pure water at 2 bar until the water flux remained constant with time. As illustrated in Figure 2, 30 mL enzyme solutions (0.5 mg of laccase or 1 mg of ADH) were added into the cell with the washed membranes for the enzyme immobilization operations at 2 bar and 100 rpm agitation. The permeate was collected in precision cylinders for analysis. When 28 mL of liquid had passed through the membranes (P), the filtration was stopped and the enzyme-loaded membranes were washed with 15 mL of pure water at 2 bar. The final retentate and washing residual (W, 17 mL) were combined in order to calculate the amount of immobilized enzyme by mass balance. Then, on the basis of previous studies,[18−20,23,26] a 10 mL dopamine (2 g L^{-1}) solution was poured into the open cell and the coating was carried out at 100 rpm agitation and room temperature for different times (Figure 2). The coating was accomplished by maintaining the system at pH 8.5 to allow the polymerization of the dopamine[16,26] except where otherwise stated. After coating, the membranes were cleaned using pure water at 2 bar for 30 min and the permeability was also measured. The coated membranes (uncoated membranes were used as a control) were removed from the cell and immersed in 10 mL of phosphate buffer (pH 7) overnight. Subsequently, the membranes were mounted in the cell in normal mode (skin layer facing feed without extra support) and washed with 15 mL of pure water at 2 bar. The buffer used for soakage and the water used for washing (SW, 25 mL) were collected and analyzed in order to measure the enzyme leakage. Finally, the substrate was added to the cell and the enzyme activity was evaluated via the conversion of the permeate using a flow-through mode (Figure 2).

2.3. Determination of Enzyme Loading. The concentration of the enzyme was measured as a protein concentration using the Bradford assay with a spectrophotometer (PerkinElmer lambda20 UV/vis, Germany). The amount of immobilized enzyme was calculated from the mass balance equation:

$$M_{IMM} = M_T - C_P \times V_P - C_W \times V_W - C_{SW} \times V_{SW} \tag{1}$$

where M_{IMM} and M_T are immobilized and total enzyme amounts, respectively; C_P and V_P are the enzyme concentration and volume in the permeate, respectively; C_W and V_W are the enzyme concentration and volume in the mixture of retentate and washing residual (before coating), respectively; C_{SW} and V_{SW} are the enzyme concentration and volume in the mixture of soaking and washing residual (after membrane reversal), respectively.

2.4. Activity Assay of Free and Immobilized Enzymes. The activity of free and immobilized laccase was determined using ABTS as the substrate (0.05 mM, pH 5.2) at room temperature. For the free laccase, 50 μL of enzyme solution (0.025 g L^{-1}) was added into a cuvette with 3 mL of ABTS substrate, and the oxidation rate of ABTS to $ABTS^+$ was monitored by measuring absorbance at 420 nm. The reaction solution was mixed quickly, and the absorbance was recorded every 5 s (total time = 2 min). For the immobilized laccase, 50 mL of ABTS substrate was filtrated using the enzyme-loaded membranes at 2 or 4 bar. The permeate was collected every 4 mL, and the absorbance at 420 nm was measured immediately. The enzyme activity was estimated as the conversion rate of ABTS in the permeate:

$$\text{conversion}\,(\%) = \left(\frac{A_p}{A_{max}}\right) \times 100 \tag{2}$$

where A_p and A_{max} are the absorbance at 420 nm for the permeate and the feed solution after full oxidation ($ABTS^+$), respectively.

The stability of free and immobilized enzymes was expressed as relative activity (%):

$$\text{relative activity}\,(\%) = \frac{X_t}{X_i} \times 100$$

where X_i and X_t are the oxidation rate or conversion in the initial and final measurements, respectively.

The activity of immobilized ADH was determined using the conversion of formaldehyde (HCOH) to methanol (CH_3OH) with oxidation of NADH to NAD^+, as described in previous work.[4,15] The substrate solution contained 1 mM formaldehyde and 0.1 mM NADH prepared in a Tris-HCl buffer (pH 7.0). After filtration, the remaining substrate in the retentate was also measured, which showed that the reaction in the bulk solution was negligible.

2.5. Stability of Biocatalytic Membranes (RC10 kDa). The stability of biocatalytic membranes was investigated in three series.

2.5.1. Effect of Storage and Reuse. The biocatalytic membranes with and without dopamine coating were stored in the fridge (4 °C) in phosphate buffer (pH 7) and examined periodically. The free enzyme was also stored and tested at the same conditions but without reuse.

2.5.2. Effect of Temperature and pH. Free enzyme and biocatalytic membranes were stored at pH 5 and different temperatures (25 or 50 °C) and examined periodically. They were also incubated in different buffer solutions (pH = 3, 5, 7, 8.4, and 10.2) for 1.5 h at room temperature and then tested at pH 5.2. New biocatalytic membranes were used for each experiment.

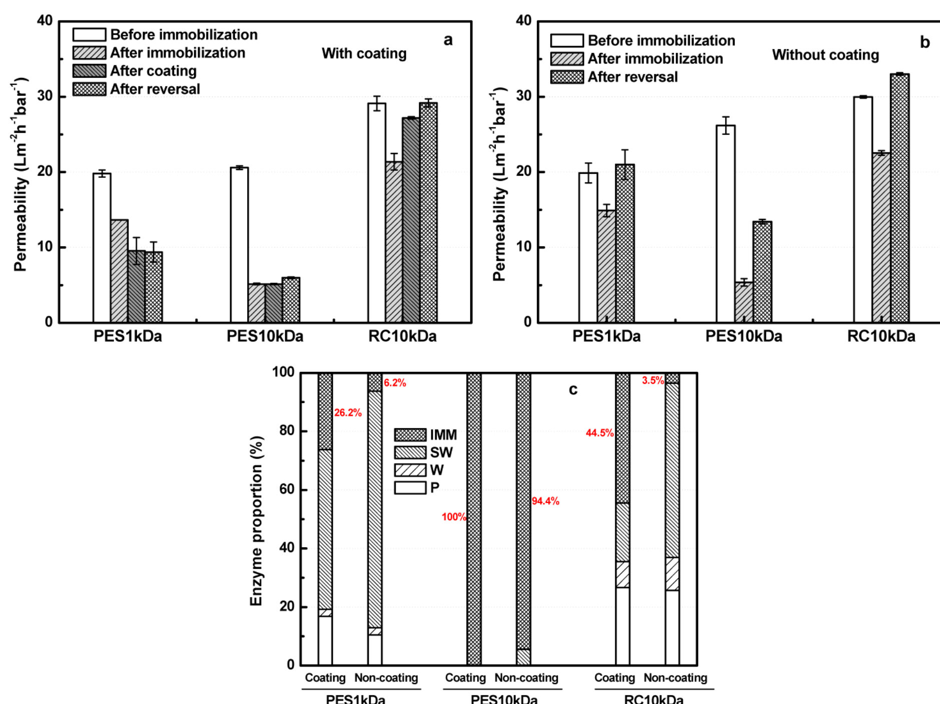


Figure 3. Membrane permeability variations in the different operating stages (a) with and (b) without dopamine coating; (c) enzyme loading efficiency and enzyme loss in the residues (IMM: immobilized enzyme; SW: enzyme residues during soakage and washing in normal mode; W: enzyme residues during washing in reverse mode; P: enzyme residues in the permeate during immobilization). Laccase was immobilized at pH = 5.2, coating pH = 8.5, and coating time = 5 h.

2.5.3. Effect of Hydrodynamic and Chemical Cleaning. The biocatalytic membranes with dopamine coating were washed using pure water at a pressure of 2 bar, and then, the pressure was suddenly released. The pressure shock operation was used to mimic back-flushing for hydrodynamic cleaning. After the pressure shock, the membrane permeability and activity of immobilized enzyme were measured using 15 mL of ABTS as substrate. This procedure was repeated ten times. In addition, the biocatalytic membrane was cleaned by acidic (pH 1.5) or alkaline (pH 11.5) solutions prepared using HCl or NaOH. The cleaning lasted for 10 min without pressure at 100 rpm agitation. Then, the membranes were rinsed using pure water and washed by 50 mM phosphate buffer (pH 7) for 5 min at a pressure of 2 bar. Afterward, the activity of immobilized enzyme was measured at pH 5.2 with 100 mL of ABTS substrate (two cycles). New biocatalytic membranes were used for each experiment.

2.6. Membrane and Polydopamine Characterization. *2.6.1. Scanning Electron Microscopy (SEM).* All membranes were immersed in liquid nitrogen and held in liquid nitrogen while cutting them with a precooled pair of scissors. Next, the sample was left to dry in ambient conditions. When dry, the sample was mounted onto a slotted aluminum stub and grounded with copper tape. Finally, the surface was coated with 2 nm of platinum in a Cressington 208HR High Resolution Sputter Coater. The scanning electron micrographs were acquired with an FEI Helios dual beam microscope. The secondary electron signal was collected at 3 keV and 26 pA with an Everhart-Thornley detector and/or through a Lens detector as required.

2.6.2. Transmission Electron Microscopy (TEM). For TEM analysis, 200 mesh Cu C lacey grids were used. First, the grids were exposed to an argon oxygen mixture glow discharge in a Cressington 208HR High Resolution Sputter Coater (two times 12 s at 5 V) in order to increase the polydopamine adhesion. Second, a droplet of polydopamine solution was added to the grid and left to settle for 1 min. The grid with polydopamine was then washed in pure water three times, followed by staining in 2% uranyl acetate for 1 min. The excess stain was washed three times in pure water. Finally, the grid with the sample was wicked from the sides with a Watman filter paper and left to dry in ambient conditions for a few minutes. The TEM micrographs were acquired using an FEI Tecnai T20 G2 transmission electron microscope at 200 keV in bright field mode.

3. RESULTS AND DISCUSSION

3.1. Effect of Membrane Properties. One nanofiltration (NF) and two UF membranes were selected to verify the enzyme immobilization strategy. As seen in Figure 3a, the membrane permeability declined significantly after laccase immobilization for all the membranes due to formation of a fouling layer (i.e., pore blocking and particle deposition). The permeate flux decline caused by fouling formation during the reverse filtration is also shown in Figure S1, Supporting Information. However, after dopamine coating (membrane images are shown in Figure S2, Supporting Information) and membrane reversal, the permeability of the RC10 kDa membrane could be recovered, but permeability remained low for the other two PES membranes (Figure 3a). The differences in recoverability of permeability of the RC10 kDa membrane compared to the other membranes could be due to different mechanisms: first, as shown by our previous study,[15] fouling mechanisms are different for RC and PES membranes due to the different hydrophilicity[29] and pore structures of the membranes (see Figure 1). Pore blocking was the main fouling mechanism for PES membranes, i.e., (a) less hydrophilicity resulted in more enzyme adsorption on the pore wall and (b) more enzymes could enter deep into the finger-like sublayer, while cake layer fouling formation was dominant for RC membranes, i.e., (a) high hydrophilicity minimized the hydrophobic adsorption and (b) enzymes could be well distributed in or on the sponge-like sublayer. Second, the protein cake layer on the RC membrane caused a reduction in membrane hydrophilicity (the contact angle of the support layer increased to 96.0° ± 1.5° from 89.7° ± 1.1°), which could be mostly recovered by the dopamine coating (which is very

hydrophilic,[18,21] and the contact angle decreased to 17.8° ± 2.5°). Such permeability loss resulting from pore blocking of the PES membrane could not be regained by the hydrophilic coating. Third, after membrane reversal, the pore fouling caused by laccase was not removed by back-flushing because the dopamine coating layer blocked the "outlet", and thus, the permeability did not increase for the PES membranes. This hypothesis can be confirmed by the control experiments without dopamine coating (Figure 3b), showing that, after membrane reversal, the permeability increased significantly for all the membranes due to the massive enzyme leakage, especially for the PES1 kDa and RC10 kDa membranes.

Figure 3c shows the enzyme loading and loss during immobilization and subsequent washing for the different membranes. The enzyme loss during reverse filtration was almost zero for the PES10 kDa membrane, indicating that all the laccase could be immobilized in the membrane by entrapment and adsorption as this membrane had larger pore size (more adsorption sites and occupancy space) and relatively hydrophobic properties. Unexpectedly, the enzyme loss for the PES1 kDa membrane was higher than that for PES10 kDa, and this was presumably caused by the smaller pore size in the sublayer of the PES1 kDa membrane and resulting reduction in the number of adsorption sites and occupancy space (some enzymes could leak from the membrane via the margin of the support layer). For the RC10 kDa membrane, the enzyme loss during reverse filtration was the highest due to its high hydrophilicity (therefore super low adsorption ability[30]) and large pore size (resulting in easy leakage of enzymes from the membrane[15]). Regarding the enzyme loading, the PES10 kDa membrane was the best, even without the dopamine coating (~94%), meaning that hydrophobic adsorption in the pores was the main fouling mechanism and that it was irreversible to hydrodynamic cleaning. However, for the other two membranes, the improvement of the enzyme loading by the dopamine coating was significant (from 6.2% to 26.2% for the PES1 kDa and from 3.5% to 44.5% for the RC10 kDa). Apparently, the enzyme leakage during overnight soaking and washing in the normal filtration (SW) contributed the most to the total enzyme loss, and dopamine coating greatly decreased such enzyme leakage, especially for the RC10 kDa membrane (from 60% to 20%) (Figure 3c). The enzyme leakage (SW) was much lower for the RC10 kDa membrane with coating than the PES1 kDa membrane with coating due to the larger pore size of the RC10 kDa membrane, indicating that more enzymes could enter into the porous sublayer of the RC10 kDa membrane and were then wrapped by the polydopamine "network". The hypothesis that the dopamine self-polymerized and produced a cover layer for the enzymes in the porous sublayer was verified by SEM and TEM images of polydopamine morphology on lacey carbon film (Figure 4). If the enzymes are deposited/adsorbed in the pores or on the "basins" of membrane surface during the reverse filtration (Figure 2, left), the dopamine coating would immobilize them by entrapment when the membrane was switched to normal mode (Figure 2, right). Even though the enzyme loading was the highest for the PES10 kDa membrane (100%), the permeability was extremely low (6 L m^{-2} h^{-1} bar^{-1}) and the trade-off between enzyme loading and membrane permeability was evident; for the PES1 kDa membrane with dopamine coating, the values were 26.2% and 12 L m^{-2} h^{-1} bar^{-1}, respectively (Figure 3). The RC10 kDa membrane with dopamine coating had a relatively high enzyme loading (44.5%) and super high permeability (29 L m^{-2} h^{-1} bar^{-1}), which defied the trade-off between enzyme loading and permeability and was therefore considered a desirable choice for laccase immobilization. When the pH during immobilization was changed to 7.0, although the improvement of membrane permeability and enzyme loading with the dopamine coating was still obvious, the achievement was much lower than for immobilization at pH 5.2 because many more enzymes leaked during the reverse filtration at pH 7.0 (before coating) (Figure S3, Supporting Information). The high enzyme leakage during reverse filtration could be explained by the fact that, with an increase in pH, generally the polymeric membranes would become more charged and more hydrophilic, leading to stronger antifouling performances and resulting in less "fouling" formation due to adsorption of enzymes in the membranes at higher pH.[30−32] Figure S4, Supporting Information, also shows the cross-section images of membranes before and after enzyme immobilization plus dopamine coating, indicating that there is no significant change in pore structure by enzymes and polydopamine depositions.

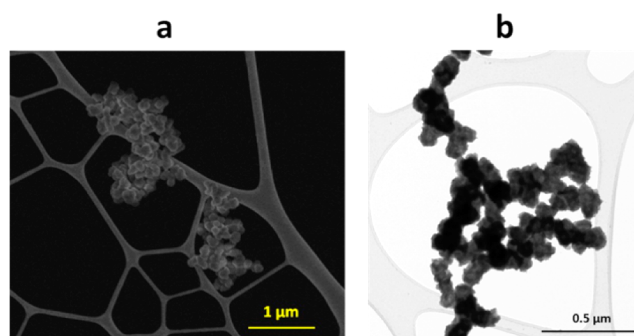


Figure 4. Morphology of polydopamine on lacey carbon film: (a) SEM image and (b) TEM image.

Figure 5 shows the ABTS conversion in flow-through mode and the permeate flux in the different biocatalytic membranes. For the PES1 kDa membrane, although the conversion with dopamine coating was much higher than that without coating due to the differences in enzyme loading, the permeate flux was much lower for the membrane with the dopamine coating (60% reduction). For the PES10 kDa membrane, the conversion was similar for the membranes with and without the dopamine coating due to the high enzyme loading for both, but the permeate flux was significantly decreased (by 63%) after coating. For the RC10 kDa membrane, since the enzyme loading was largely improved by dopamine coating, the conversion was also greatly increased but the permeate flux did not show an obvious decline, which is most desirable for a biocatalytic membrane. It is worth mentioning that, when the permeate flux increased at higher applied pressures, the conversion only decreased accordingly in some cases (Figure 5). Theoretically, the contact time between enzymes and substrate will decrease with increasing permeate flux, and thus, the conversion should be reduced at increased permeate fluxes. However, for the RC10 kDa membrane with the dopamine coating and the PES10 kDa membranes without coating, there was little change in conversion when the permeate flux was doubled, which was caused by excessive enzyme loading for the low substrate concentration tested and the high catalytic efficacy of the enzymes. Another interesting phenomenon was that the conversion sharply increased during the first cycle for all the membranes with a coating, which could be caused by the reversible suppression of laccase activity that occurred during

dx.doi.org/10.1021/am507308k | ACS Appl. Mater. Interfaces 2014, 6, 22894−22904

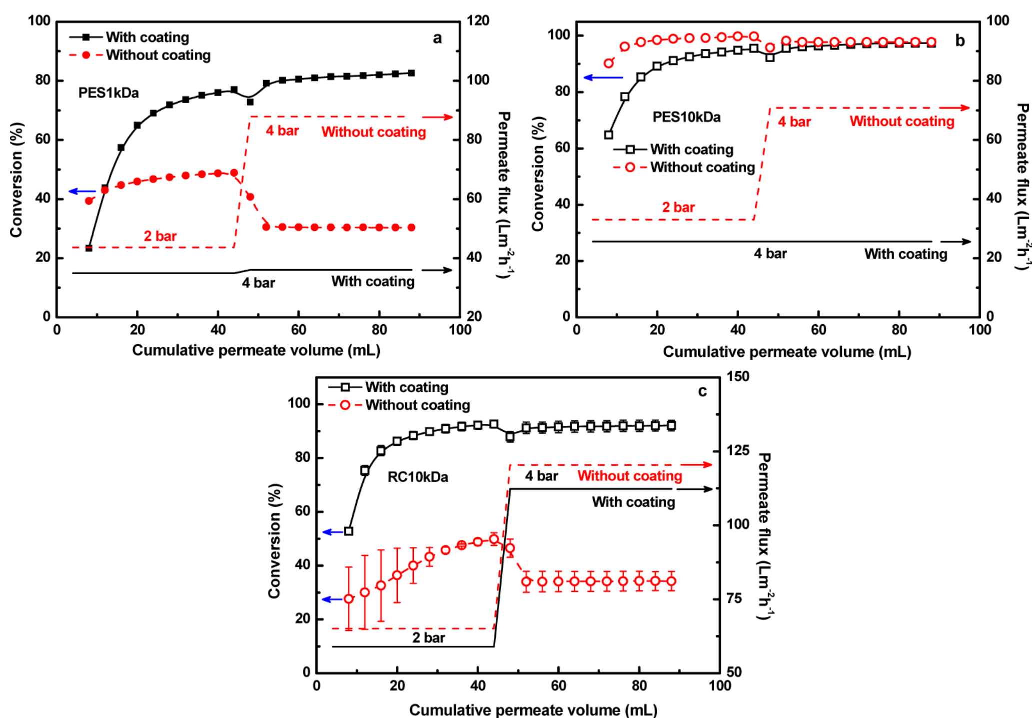


Figure 5. ABTS conversion in flow-through mode and permeate flux by different biocatalytic membranes of (a) PES1 kDa, (b) PES10 kDa, and (c) RC10 kDa with or without dopamine coating. Laccase was immobilized at pH = 5.2, coating pH = 8.5, and coating time = 5 h.

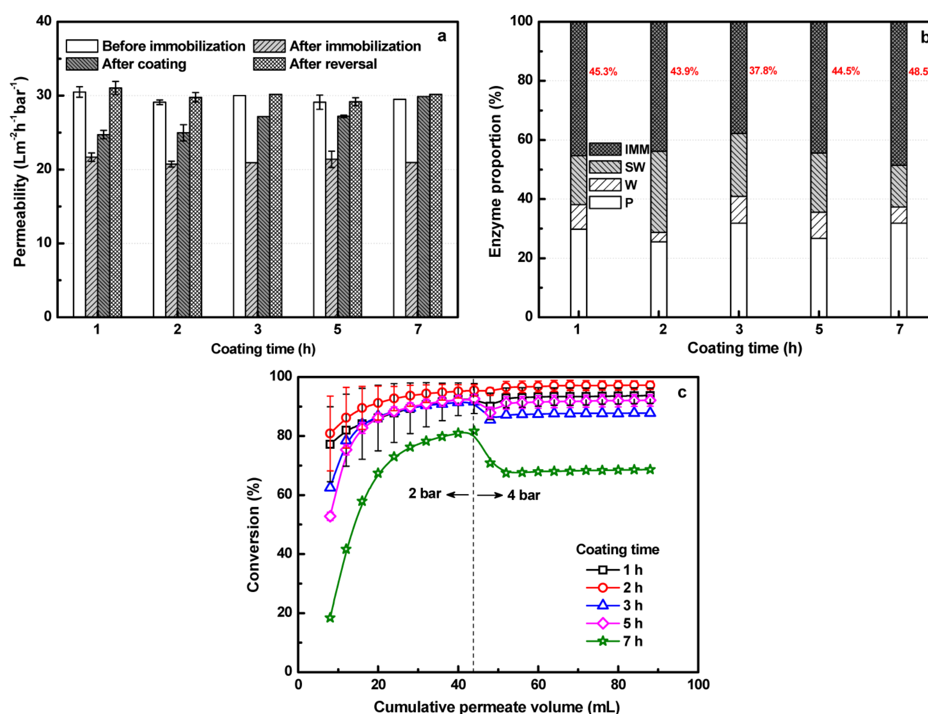


Figure 6. Effect of coating time on (a) permeability variations, (b) enzyme loading and enzyme loss in different stages, and (c) ABTS conversion in flow-through mode. Laccase was immobilized at pH = 5.2 in RC10 kDa membranes. Coating pH = 8.5.

the alkaline coating pH (i.e., 8.5)[12] and the subsequent recovery at pH 5.2 during the reaction. According to the results in Figure 5, the conversion was directly proportional to the enzyme loading, revealing that most of the activity of the immobilized enzymes was retained by this noncovalent immobilization strategy. A similar conclusion also could be obtained by the results in Figure S5, Supporting Information (where the immobilization pH was 7.0). Thanks to its high permeability and enzyme loading, the RC10 kDa membrane was selected for the following studies.

3.2. Effect of Coating Time. As mentioned above, laccase activity was probably depressed by the alkaline pH during coating, and thus, it would be desirable to shorten the dopamine coating time while maintaining the enzyme loading.

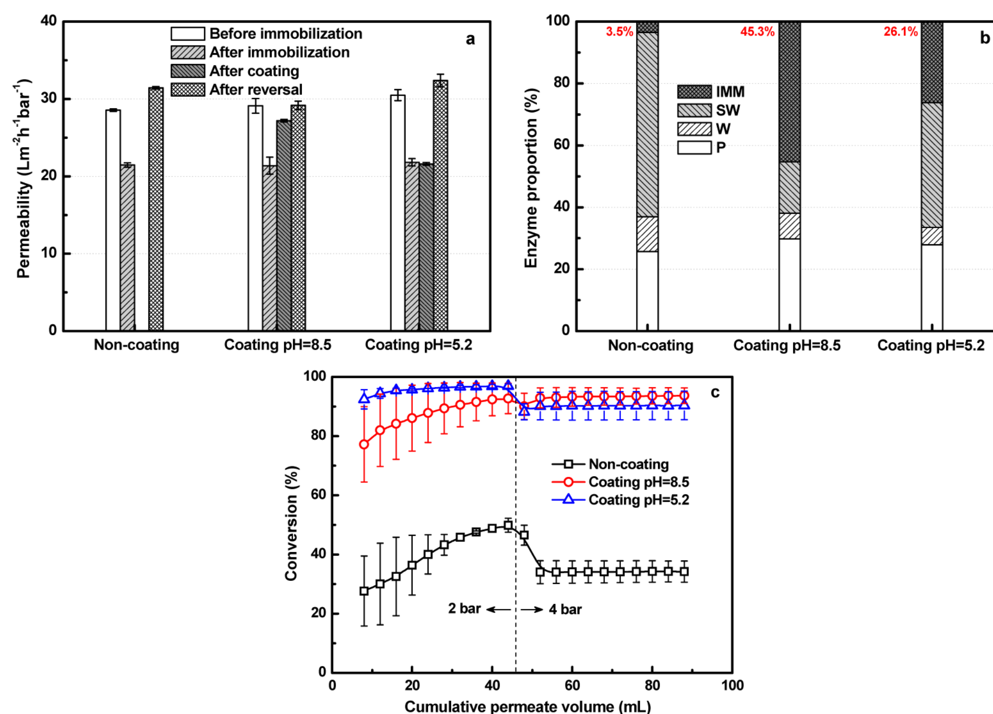


Figure 7. Effect of coating pH on (a) permeability variations, (b) enzyme loading and enzyme loss in different stages, and (c) ABTS conversion in flow-through mode. Coating time = 1 h. Laccase was immobilized at pH = 5.2 in RC10 kDa membranes.

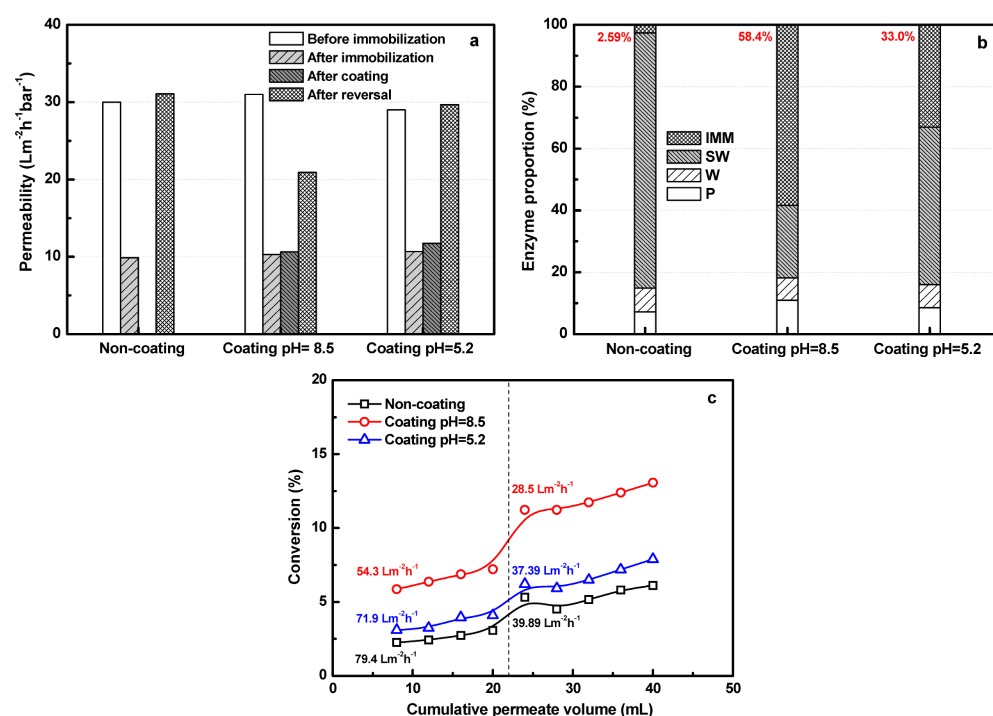


Figure 8. Effect of coating pH on (a) permeability variations, (b) enzyme loading and enzyme loss in different stages, and (c) ABTS conversion in flow-through mode. Coating time = 1 h. Alcohol dehydrogenase was immobilized at pH = 5.2 in RC10 kDa membranes.

As seen in Figure 6a, the membrane permeabilities after immobilization are very similar for different coating times, while the permeability after coating increased with coating time, which is not in accordance with the other studies on membrane surface modification by dopamine coating.[18−20] The observed increase in permeability with coating time implies that the dopamine coating did not form a dense layer on the membrane sublayer (i.e., the hydraulic resistance of the coated membrane did not increase because the membrane permeabilities after reversal were similar for different coating times, see Figure 6a), even though the hydrophilicity of the membrane sublayer increased with increasing coating time. It could be speculated that the dopamine polymerization was not as efficient in the membrane as on the membrane surface because the oxygen transport to the dopamine was limited by the porous support

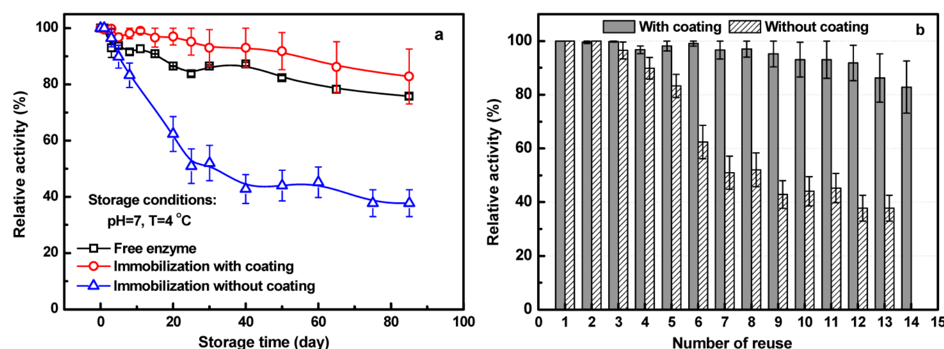


Figure 9. Relative activity of free and immobilized laccase with (a) storage time and (b) reuse cycle. Free enzyme and biocatalytic membranes (RC10 kDa) were stored in the fridge with phosphate buffer (pH = 7).

layer. Moreover, an increase in coating time did not increase the enzyme loading, as shown in Figure 6b.

The dopamine coating indeed had a negative effect on laccase activity (Figure 6c) as the "wake up" time of immobilized laccase increased with increasing coating time. Hence, when the coating time reached 7 h, the coating process apparently damaged the laccase irreversibly and the achieved extent of conversion was lower than that of the membranes with shorter coating times, although the enzyme loadings were similar. Therefore, a coating time of 1 h was selected for the subsequent investigations.

3.3. Effect of Coating pH. It is well-known that laccase can catalyze the polymerization of dopamine at acidic pH.[27,33] In order to clarify which polymerization approach was more suitable to immobilize laccase in the membrane sublayer, the effect of coating pH on membrane permeability, enzyme loading, and activity was investigated. Coating with dopamine at pH 5.2 did not increase the membrane permeability (Figure 7a), possibly because the dopamine coating catalyzed by laccase (i.e., laccase can catalyze dopamine polymerization) is not thought to increase the membrane hydrophilicity.[27] The hypothesis to explain this observation is that dopamine polymerization and adhesion/deposition on the membrane only occurs near sites where laccase is located and that the coating layer is very loose and local at such pH. This explanation is corroborated by the images shown in Figure S6, Supporting Information, which reveals that the color of the membrane after coating with dopamine at pH 5.2 was much lighter than the membrane coated with dopamine at pH 8.5. After membrane reversal, the membrane permeability increased significantly at coating pH of 5.2 and became even higher than the permeability before immobilization, equaling the membrane without coating (Figure 7a). However, as seen in Figure 7b, though the enzyme loading of the membrane with coating pH of 5.2 decreased by 42% compared to that with coating pH of 8.5, there was still 26% laccase immobilized in the membrane when the membrane was coated with dopamine at pH 5.2, which was much higher than the enzyme loading of the membrane without coating (3.5%). Moreover, as shown in Figure 7c, the ABTS conversion was higher and more stable at the beginning of the reaction using the membrane that was coated at pH 5.2, verifying again that coating at alkaline pH had a negative effect on the laccase activity. Due to the lower enzyme loading of the membrane coated at pH 5.2 compared to pH 8.5, the order of conversion shifted when the applied pressure went up. Therefore, considering that a higher enzyme loading was one of our key objectives, the self-polymerized coating of dopamine at pH 8.5 was preferred for this application when the coating time was short.

3.4. Effect of Immobilized Enzyme. In order to generalize the proposed enzyme immobilization strategy, another model enzyme, ADH, was immobilized using the same conditions used for laccase immobilization. As shown in Figure 8a, the permeability of the membranes with immobilized ADH did not increase after dopamine coating at either pH, while after membrane reversal, the permeability was recovered for the membranes without coating and also for the membrane that was coated at pH 5.2 but not for the membrane that was coated at pH 8.5. The membrane permeability results of the membranes with immobilized ADH were inconsistent with those of the membranes with immobilized laccase. First, the difference in the permeability after membrane reversal at different coating pH values was caused by their different enzyme loadings (Figure 8b). Second, the resulting dopamine coating at pH 8.5 did not improve the membrane permeability for ADH immobilization (Figure 8a), unlike dopamine coating at pH 8.5 for laccase immobilization (Figure 7a), which confirmed that the immobilized laccase enzymes indeed catalyzed the dopamine polymerization at pH 8.5. The ability of the immobilized laccase enzymes to catalyze the polymerization of dopamine at pH 8.5 was confirmed by the images displayed in Figure S6 (right), Supporting Information, where the color of the laccase-loaded membrane after coating at pH 8.5 was much darker than that of the ADH-loaded membrane. However, dopamine polymerization was not enhanced in the presence of free laccase at pH 8.5, as shown in Figure S6 (left), Supporting Information, indicating that the immobilization of laccase may have improved its stability at alkaline pH.[12] Moreover, as seen in Figure 8b, though the dopamine coating for membranes with immobilized ADH was not as intense as for membranes with immobilized laccase, the enzyme loading of membranes with immobilized ADH was higher than that obtained for the membranes with immobilized laccase (58.4% vs 45.3% at pH 8.5; 33.0% vs 26.1% at pH 5.2). The high enzyme loading of the membranes with immobilized ADH is assumed to be attributed to the larger molecular weight of ADH (141 kDa) compared to laccase (60−70 kDa), resulting in less enzyme leakage during the reverse filtration (Figure 8b). It is worth mentioning that the dopamine coating occurred at pH 5.2 for membranes with immobilized ADH because the enzyme loading increased to 33% from 2.6% compared to the membrane without coating, although the ADH probably could not catalyze the dopamine polymerization and the self-polymerization of dopamine is expected to be extremely slow at pH 5.2 (see Figure S6 (left), Supporting Information). As

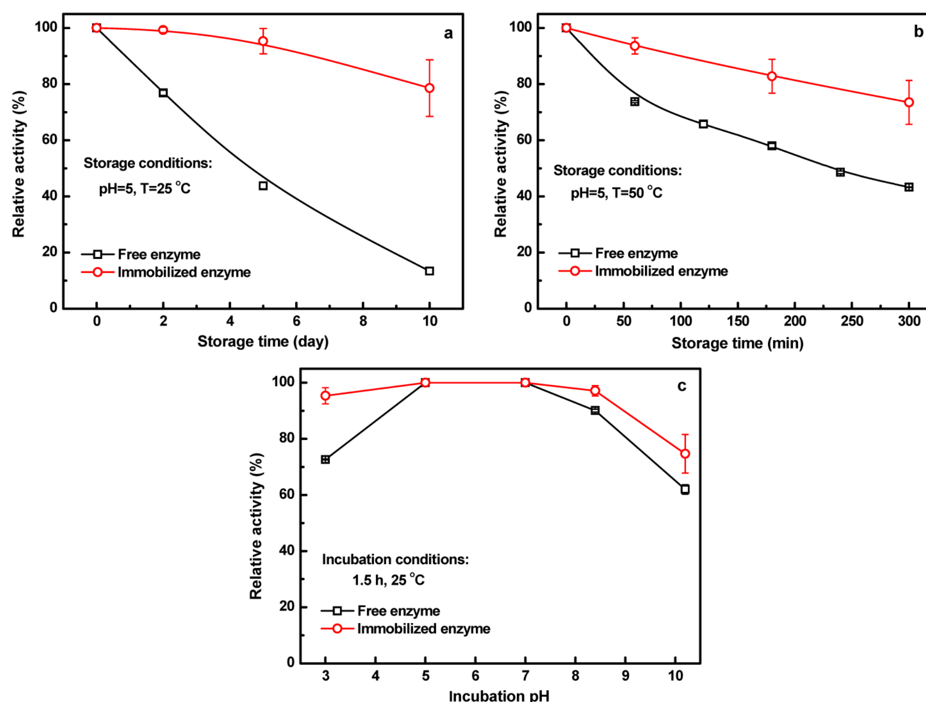


Figure 10. Effect of (a, b) storage temperature and (c) incubation pH on laccase stability for free enzyme and biocatalytic membrane (RC10 kDa with laccase and dopamine coating).

shown by Figures 7b and 8b, the enzyme loading of membranes with immobilized ADH and coating at pH 8.5 was much higher than at pH 5.2, suggesting that the dopamine coating should be carried out at alkaline pH for all the enzymes. Although the dopamine would deposit on the membrane at pH 5.2, the efficiencies of enzyme immobilization and permeability improvement were not satisfactory. Regarding the activity of immobilized ADH (Figure 8c), the conversion was higher for the membrane with higher enzyme loading, but such promotion was limited, and the maximum conversion was below 15% even with 43.6 μg cm^{-2} of ADH in the membrane. The observed low conversion of ADH allows us to conclude that the dopamine coating resulted in a significant activity loss of ADH (maybe reversible); hence, this strategy was not suitable for the immobilization of ADH.

3.5. Stability of Biocatalytic Membranes. *3.5.1. Effect of Storage and Reuse.* It is well-known that diffusion barrier and enzyme leakage are the two main drawbacks for enzyme immobilization by entrapment. The diffusion barrier can be partially overcome by the use of flow-through operation with a biocatalytic membrane. As for the enzyme leakage, the stability of the immobilized laccase during storage and reuse was investigated to know whether it could be avoided by dopamine coating. As shown in Figure 9, the relative activity of the immobilized laccase with dopamine coating is much more stable than that without coating, and it is even higher than that of free laccase without reuse, which suggests that the dopamine coating can completely wrap the laccase and fully eliminate enzyme leakage for a long-term operation. A slight decline in relative enzyme activity with time for the membrane with immobilized laccase and dopamine coating was possibly caused by the enzymes inherent activity loss with time, which also occurred in the free laccase without reuse (pH 7, temperature 4 °C). After 85 days of storage and reuse 14 times, more than 80% of the enzymatic activity was retained by the biocatalytic membrane with dopamine coating, while the relative activity was less than 40% without the coating.

3.5.2. Effect of Temperature and pH. Enzyme stability is sensitive to pH and temperature. After storage for 10 days at pH 5 and room temperature (25 °C), the free laccase lost more than 80% of activity even without reuse, while the immobilized laccase retained around 80% of the initial activity with reuse four times (Figure 10a). When the storage temperature was increased to 50 °C, the relative activity decreased to 43% in 5 h for free laccase while it was maintained at 74% for the immobilized laccase (Figure 10b). The free and immobilized enzymes were also incubated at different pH for 1.5 h (25 °C), and then, their activity was measured at the same conditions (pH 5.2). As seen in Figure 10c, the improvement in the relative activity by laccase immobilization was significant for the incubation at pH 3, 8.4, and 10.2. Therefore, for laccase, the resistance against high temperature and acidic/alkaline pH was enhanced by this immobilization strategy, which might be due to conformational changes of the enzyme caused by interaction with the membrane and polydopamine, as well as the decrease in molecular mobility.[34]

3.5.3. Effect of Hydrodynamic and Chemical Cleaning. For "flow-through" EMRs, membrane fouling is inevitable and membrane cleaning is required for a long-term run. Generally, chemical cleaning is the conventional cleaning method used in industry,[35] but it cannot be recommended for biocatalytic membranes because cleaning agents may produce permanent damage to the enzyme or release the immobilized enzymes (by changing membrane structure or breaking bonds). Back-flushing operation is also an effective membrane cleaning method,[36] but it may induce enzyme leakage by deforming the membrane. There are very few studies on this topic. For this reason, we evaluated the mechanical resistance of the selected biocatalytic membrane (RC10 kDa with laccase and dopamine coating) to pressure shock and also its chemical resistance to

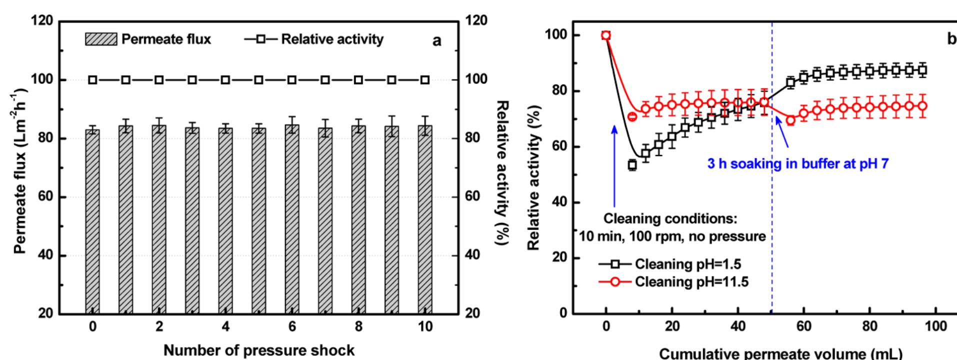


Figure 11. Effect of (a) pressure shock (mimicking back-flushing operation) and (b) chemical cleaning on the stability of biocatalytic membranes (RC10 kDa with laccase and dopamine coating).

acidic/alkaline cleaning. As shown in Figure11a, the permeate flux through the biocatalytic membrane and its relative activity did not change during 10 cycles of pressure shock, indicating that the membrane skin layer can efficiently reject laccase and prevent enzyme leakage during back-flushing. However, chemical cleaning had a negative effect on enzyme activity. The relative activity thus declined to 53% after acidic cleaning (pH 1.5) for 10 min (Figure 11b). It was also found that part of the activity loss was reversible, and after more than a 3 h soaking in buffer at pH 7, the relative activity was restored to 88%, while alkaline cleaning (pH 11.5) could irreversibly eliminate more than 25% of the initial activity of the biocatalytic membrane. Therefore, alkaline cleaning produced more permanent activity loss than acidic cleaning, and chemical cleaning of the biocatalytic membrane at extreme pH should be avoided.

4. CONCLUSION

This work confirmed that high performance biocatalytic membranes could be prepared using fouling-induced enzyme immobilization and subsequent dopamine coating. The data confirmed that the dopamine produced a surface coating that entrapped the enzymes in the membrane sublayer, even after membrane reversal. Through this strategy, the membrane sublayer was functionalized as a catalytically active layer, and at the same time, both the separation capacity of the skin layer and its antifouling ability were maintained. The study shows that the material, pore size, and structure of the membrane have a significant effect on the resulting enzyme loading and final membrane permeability of the biocatalytic membrane. Using a highly hydrophilic RC10 kDa membrane with a sponge-like sublayer, the typical trade-off between enzyme loading and permeability was broken, and laccase loading reached 44.5% without any permeability loss.

Regarding the membrane permeability enhancement and high enzyme loading caused by dopamine coating, laccase-catalyzed dopamine polymerization at pH 5.2 was not as efficient as dopamine self-polymerization at pH 8.5, while the dopamine coating on the laccase-loaded membrane at pH 8.5 involved both polymerization mechanisms. ADH could also be immobilized in the membrane using this strategy, no matter whether the coating pH was 5.2 or 8.5 (the loading was 33% and 58%, respectively), indicating that this noncovalent enzyme immobilization in the membrane sublayer with dopamine coating could be extrapolated to a number of enzymes. After 85 days of storage and reuse 14 times, more than 80% of the laccase activity was retained for the membrane with immobilized laccase and dopamine coating, while the relative activity was less than 40% without the coating. For the membrane with immobilized laccase and dopamine coating, the resistance against high temperature and acidic/alkaline pH was improved. Moreover, such biocatalytic membranes could resist mild hydrodynamic cleaning (e.g., back-flushing), while the catalytic ability was reduced by chemical cleaning at extreme pH (e.g., 1.5 and 11.5). Since the immobilized enzyme does not directly face the bulk of EMRs and the substrate can be specifically selected by the separation skin layer, this biocatalytic membrane is promising for applications in cascade catalytic reactions.

■ ASSOCIATED CONTENT

Supporting Information

Permeate flux during reverse filtration of enzyme and membrane images after coating. Membrane permeability, enzyme loading, and catalytic performance for different membranes when laccase was immobilized at pH 7. SEM images of cross-section of membranes before and after enzyme immobilization plus dopamine coating. Observations of dopamine oxidation and membrane image after coating at different conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: mp@kt.dtu.dk. Tel.: +45 4525 2950. Fax: +45 4588 2258.
*E-mail: Jluo@kt.dtu.dk. Tel.: +45 4525 2950. Fax: +45 4588 2258.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Franssen, M. C. R.; Steunenberg, P.; Scott, E. L.; Zuilhof, H.; Sanders, J. P. M. Immobilised Enzymes in Biorenewables Production. *Chem. Soc. Rev.* **2013**, *42*, 6491−6533.

(2) Sheldon, R. A.; van Pelt, S. Enzyme Immobilisation in Biocatalysis: Why, What and How. *Chem. Soc. Rev.* **2013**, *42*, 6223−6235.

(3) Zeuner, B.; Luo, J.; Nyffenegger, C.; Aumala, V.; Mikkelsen, J. D.; Meyer, A. S. Optimizing the Biocatalytic Productivity of an Engineered Sialidase from Trypanosoma Rangeli for 3′-Sialyllactose Production. *Enzyme Microb. Technol.* **2014**, *55*, 85−93.

(4) Luo, J.; Meyer, A. S.; Jonsson, G.; Pinelo, M. Fouling-Induced Enzyme Immobilization for Membrane Reactors. *Bioresour. Technol.* **2013**, *147*, 260−268.

(5) Rios, G. M.; Belleville, M. P.; Paolucci, D.; Sanchez, J. Progress in Enzymatic Membrane Reactors – A Review. *J. Membr. Sci.* **2004**, *242*, 189–196.
(6) Giorno, L.; Drioli, E. Biocatalytic Membrane Reactors: Applications and Perspectives. *Trends Biotechnol.* **2000**, *18*, 339–349.
(7) Jochems, P.; Satyawali, Y.; Diels, L.; Dejonghe, W. Enzyme Immobilization on/in Polymeric Membranes: Status, Challenges and Perspectives in Biocatalytic Membrane Reactors (BMRs). *Green Chem.* **2011**, *13*, 1609–1623.
(8) Ameur, S. B.; Luminiţa Gîjiu, C.; Belleville, M.-P.; Sanchez, J.; Paolucci-Jeanjean, D. Development of a Multichannel Monolith Large-Scale Enzymatic Membrane and Application in an Immobilized Enzymatic Membrane Reactor. *J. Membr. Sci.* **2014**, *455*, 330–340.
(9) Lante, A.; Crapisi, A.; Krastanov, A.; Spettoli, P. Biodegradation of Phenols by Laccase Immobilised in a Membrane Reactor. *Process Biochem.* **2000**, *36*, 51–58.
(10) Georgieva, S.; Godjevargova, T.; Mita, D. G.; Diano, N.; Menale, C.; Nicolucci, C.; Carratelli, C. R.; Mita, L.; Golovinsky, E. Non-Isothermal Bioremediation of Waters Polluted by Phenol and Some of Its Derivatives by Laccase Covalently Immobilized on Polypropylene Membranes. *J. Mol. Catal. B: Enzym.* **2010**, *66*, 210–218.
(11) Chea, V.; Paolucci-Jeanjean, D.; Belleville, M. P.; Sanchez, J. Optimization and Characterization of an Enzymatic Membrane for the Degradation of Phenolic Compounds. *Catal. Today* **2012**, *193*, 49–56.
(12) Hou, J.; Dong, G.; Ye, Y.; Chen, V. Laccase Immobilization on Titania Nanoparticles and Titania-Functionalized Membranes. *J. Membr. Sci.* **2014**, *452*, 229–240.
(13) Hou, J.; Dong, G.; Ye, Y.; Chen, V. Enzymatic Degradation of Bisphenol-A with Immobilized Laccase on TiO_2 Sol–Gel Coated PVDF Membrane. *J. Membr. Sci.* **2014**, *469*, 19–30.
(14) Giorno, L.; Drioli, E.; Carvoli, G.; Cassano, A.; Donato, L. Study of an Enzyme Membrane Reactor with Immobilized Fumarase for Production of L-Malic Acid. *Biotechnol. Bioeng.* **2001**, *72*, 77–84.
(15) Luo, J.; Meyer, A. S.; Jonsson, G.; Pinelo, M. Enzyme Immobilization by Fouling in Ultrafiltration Membranes: Impact of Membrane Configuration and Type on Flux Behavior and Biocatalytic Conversion Efficacy. *Biochem. Eng. J.* **2014**, *83*, 79–89.
(16) Liu, Y.; Ai, K.; Lu, L. Polydopamine and Its Derivative Materials: Synthesis and Promising Applications in Energy, Environmental, and Biomedical Fields. *Chem. Rev.* **2014**, *114*, 5057–5115.
(17) Arena, J. T.; McCloskey, B.; Freeman, B. D.; McCutcheon, J. R. Surface Modification of Thin Film Composite Membrane Support Layers with Polydopamine: Enabling Use of Reverse Osmosis Membranes in Pressure Retarded Osmosis. *J. Membr. Sci.* **2011**, *375*, 55–62.
(18) Cheng, C.; Li, S.; Zhao, W.; Wei, Q.; Nie, S.; Sun, S.; Zhao, C. The Hydrodynamic Permeability and Surface Property of Polyethersulfone Ultrafiltration Membranes with Mussel-Inspired Polydopamine Coatings. *J. Membr. Sci.* **2012**, *417–418*, 228–236.
(19) McCloskey, B. D.; Park, H. B.; Ju, H.; Rowe, B. W.; Miller, D. J.; Freeman, B. D. A Bioinspired Fouling-Resistant Surface Modification for Water Purification Membranes. *J. Membr. Sci.* **2012**, *413–414*, 82–90.
(20) Kasemset, S.; Lee, A.; Miller, D. J.; Freeman, B. D.; Sharma, M. M. Effect of Polydopamine Deposition Conditions on Fouling Resistance, Physical Properties, and Permeation Properties of Reverse Osmosis Membranes in Oil/Water Separation. *J. Membr. Sci.* **2013**, *425–426*, 208–216.
(21) Li, Y.; Su, Y.; Zhao, X.; He, X.; Zhang, R.; Zhao, J.; Fan, X.; Jiang, Z. Antifouling, High-Flux Nanofiltration Membranes Enabled by Dual Functional Polydopamine. *ACS Appl. Mater. Interfaces* **2014**, *6*, 5548–5557.
(22) Yang, H.-C.; Pi, J.-K.; Liao, K.-J.; Huang, H.; Wu, Q.-Y.; Huang, X.-J.; Xu, Z.-K. Silica-Decorated Polypropylene Microfiltration Membranes with a Mussel-Inspired Intermediate Layer for Oil-in-Water Emulsion Separation. *ACS Appl. Mater. Interfaces* **2014**, *6*, 12566–12572.
(23) Huang, L.; Arena, J. T.; Manickam, S. S.; Jiang, X.; Willis, B. G.; McCutcheon, J. R. Improved Mechanical Properties and Hydrophilicity of Electrospun Nanofiber Membranes for Filtration Applications by Dopamine Modification. *J. Membr. Sci.* **2014**, *460*, 241–249.
(24) Wang, X.; Jiang, Z.; Shi, J.; Zhang, C.; Zhang, W.; Wu, H. Dopamine-Modified Alginate Beads Reinforced by Cross-Linking via Titanium Coordination or Self-Polymerization and Its Application in Enzyme Immobilization. *Ind. Eng. Chem. Res.* **2013**, *52*, 14828–14836.
(25) Zhang, L.; Shi, J.; Jiang, Z.; Jiang, Y.; Qiao, S.; Li, J.; Wang, R.; Meng, R.; Zhu, Y.; Zheng, Y. Bioinspired Preparation of Polydopamine Microcapsule for Multienzyme System Construction. *Green Chem.* **2011**, *13*, 300–306.
(26) Lee, H.; Rho, J.; Messersmith, P. B. Facile Conjugation of Biomolecules onto Surfaces via Mussel Adhesive Protein Inspired Coatings. *Adv. Mater.* **2009**, *21*, 431–434.
(27) Li, D.; Luo, L.; Pang, Z.; Ding, L.; Wang, Q.; Ke, H.; Huang, F.; Wei, Q. Novel Phenolic Biosensor Based on a Magnetic Polydopamine-Laccase-Nickel Nanoparticle Loaded Carbon Nanofiber Composite. *ACS Appl. Mater. Interfaces* **2014**, *6*, 5144–5151.
(28) Chao, C.; Liu, J.; Wang, J.; Zhang, Y.; Zhang, B.; Zhang, Y.; Xiang, X.; Chen, R. Surface Modification of Halloysite Nanotubes with Dopamine for Enzyme Immobilization. *ACS Appl. Mater. Interfaces* **2013**, *5*, 10559–10564.
(29) Luo, J.; Morthensen, S. T.; Meyer, A. S.; Pinelo, M. Filtration Behavior of Casein Glycomacropeptide (CGMP) in an Enzymatic Membrane Reactor: Fouling Control by Membrane Selection and Threshold Flux Operation. *J. Membr. Sci.* **2014**, *469*, 127–139.
(30) Luo, J.; Marpani, F.; Brites, R.; Frederiksen, L.; Meyer, A. S.; Jonsson, G.; Pinelo, M. Directing Filtration to Optimize Enzyme Immobilization in Reactive Membranes. *J. Membr. Sci.* **2014**, *459*, 1–11.
(31) Mänttäri, M.; Pihlajamäki, A.; Nyström, M. Effect of pH on Hydrophilicity and Charge and Their Effect on the Filtration Efficiency of NF Membranes at Different pH. *J. Membr. Sci.* **2006**, *280*, 311–320.
(32) Hadidi, M.; Zydney, A. L. Fouling Behavior of Zwitterionic Membranes: Impact of Electrostatic and Hydrophobic Interactions. *J. Membr. Sci.* **2014**, *452*, 97–103.
(33) Tan, Y.; Deng, W.; Li, Y.; Huang, Z.; Meng, Y.; Xie, Q.; Ma, M.; Yao, S. Polymeric Bionanocomposite Cast Thin Films with in Situ Laccase-Catalyzed Polymerization of Dopamine for Biosensing and Biofuel Cell Applications. *J. Phys. Chem. B* **2010**, *114*, 5016–5024.
(34) Xu, R.; Chi, C.; Li, F.; Zhang, B. Laccase-Polyacrylonitrile Nanofibrous Membrane: Highly Immobilized, Stable, Reusable, and Efficacious for 2,4,6-Trichlorophenol Removal. *ACS Appl. Mater. Interfaces* **2013**, *5*, 12554–12560.
(35) Regula, C.; Carretier, E.; Wyart, Y.; Gésan-Guiziou, G.; Vincent, A.; Boudot, D.; Moulin, P. Chemical Cleaning/Disinfection and Ageing of Organic UF Membranes: A Review. *Water Res.* **2014**, *56*, 325–365.
(36) Srijaroonrat, P.; Julien, E.; Aurelle, Y. Unstable Secondary Oil/Water Emulsion Treatment Using Ultrafiltration: Fouling Control by Backflushing. *J. Membr. Sci.* **1999**, *159*, 11–20.