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The facile synthesis of an aldehyde-containing graft copolymer membrane for covalent protein capture with retention of protein functionality

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ABSTRACT

The immobilization of biomolecules onto an insoluble carrier surface has always been a subject of great interest to enhance their resistance to pH and temperature, which aids in an increased activity lifespan as well as easy reuse of the said biomolecules. However, traditional methods are only able to provide single-layer biomolecular binding and require multiple chemical reactions to prepare the final substrate before the immobilization can be carried out properly. Here we report a facile one-step chemical synthesis of a new aldehyde-bearing graft copolymer via atom transfer radical polymerization (ATRP) for covalent protein capture in a multilayered approach to covalently capture bovine serum albumin (BSA) onto a polymeric membrane. The resultant protein-bound membrane illustrated the retention of BSA's stereoselective discrimination ability by binding to an excess of 2 mol of tryptophan/mol of BSA and demonstrated an enantioresolution of a 0.184 mM racemic tryptophan mixture with a time-averaged-separation factor of 2.9.

1. Introduction

It is generally accepted that the covalent immobilization of biomolecules onto a water insoluble surface is one of the most stable and versatile methods known [1–5] to preserve biomolecular activity, as in the case of enzymes and proteins. Long chain aldehyde polymers like polyacrolein (PAL) can be used to covalently capture proteins with the retention of its functional ability via the formation of Schiff bases, as can be observed in the use of PAL microspheres which has been documented independently by various researchers [2–4,6]. An alternative would be the use of glutaraldehyde as a cross-linker for the covalent immobilization of proteins onto polymeric carrier surfaces bearing reactive functional groups [7,8]. Proteins have long been immobilized onto a polymeric carrier surface like that of membranes or microsphere beads via different methods like physical adsorption or covalent binding, which creates avenues for viable processes involving the use of biomolecules like that of enantioresolution [8–10] or enzymatic biocatalysis [11–13]. Therefore, the covalent immobilization of biomolecules onto a membrane attributes these biomolecules a much-needed stability and a reduced tendency to desorb for a better operational performance, as well as provide an easy scale up [14] and greater cost effectiveness [15].

It has been shown that a polypeptide like poly-L-glutamic acid (PLGA) covalently bonded to a polyvinylidene fluoride (PVDF) film provides a higher enantioselectivity than physically adsorbed PLGA due to increased hydrophobic interactions with the amino acids in the feed solution [16]. The ideal situation in membrane chromatography would hence be to fabricate a membrane that is able to bind to proteins both covalently to reduce the propensity of protein desorption and in a multilayered fashion to maximize the amount of protein that can be bound to the membrane. However, methods like vapor deposition [16] or glutaraldehyde cross-linking [17] only provides a protein binding capacity of just a single monolayer, while radical polymerization methods have been utilized in the modification of a polymeric material for multilayered protein immobilization via ion exchange and found to be effective in the enantioresolution of racemic tryptophan [18]. However, the functionalization of the polymer surface via radical polymerization traditionally contains a potpourri of reaction steps in obtaining the final reactive functional groups for protein binding [8,9,19], which complicates the entire process of synthesizing the final membrane. Here in this reaction sequence we adapt a facile single-step method to graft on a reactive functional group in a controlled manner, which can then proceed to bind with the protein covalently and uniformly over an entire mass of membrane.

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PVDF is a material that has been studied extensively and found to possess excellent chemical and thermal stability, as well as a good mechanical strength [17,18], and it contains C–F haloalkane bonds, which can be utilized for atom transfer radical polymerization (ATRP) reactions [20–23]. In addition, PVDF is soluble in organic solvents like N-methyl-2-pyrrolidone (NMP) and hence its entire bulk properties can be modified in solution phase [23] rather than just surface modification. Solution phase modification appears to be more desirable than surface modification because the grafted aldehyde chains can be found within the inner surface of pores formed during the phase inversion process [21], hence allowing for the entrapment of protein within the membrane pores as well as on the surface for a thorough chromatographic separation to occur throughout the membrane.

It is to the best of our knowledge that the graft copolymerization of vinyl aldehydes like methacrolein (MA) via ATRP has not been reported in any previous research work, and we hypothesize that aldehyde-bearing vinyl monomers like MA can be radically grafted onto PVDF via ATRP to form a graft copolymer containing reactive aldehyde groups. Here in this study we synthesize a PVDF and poly(MA) graft copolymer (PVDF–g-PMA) via ATRP in one step to capture a protein covalently onto a membrane in multilayers. The crux of this study is to investigate the feasibility of grafting a vinyl aldehyde monomer onto a PVDF backbone and not on the optimization of reaction conditions or separation performances, as it is a facile single-step synthesis which does not require the use of as many chemical reagents as has been shown in other sources [8,19]. We choose to immobilize a relatively abundant protein like BSA and investigate the ability of the immobilized BSA macroligand in the field of enantioresolution to stereoselectively discriminate between tryptophan enantiomers as shown in Fig. 1 when a tryptophan racemate permeates through the membrane.

The quantity of protein that can be immobilized onto a given amount of membrane area or mass is consequently a large determinant in the overall biomolecular separation or reaction. For example, L-tryptophan exhibits a linear binding dependence on the available quantity of serum albumins like bovine serum albumin (BSA) or human serum albumin (HSA) [24–29], while enzymatic conversions of substrate are also linearly dependent on the amount of enzyme available in the reaction as illustrated by the Michaelis–Menten equation in its most simplistic form. Therefore, it would be desirable to maximize the loading of the requisite protein onto the membrane for maximal effect, as it would be most probable that the immobilization of a protein causes it to lose a certain percentage of its activity as a free molecule.

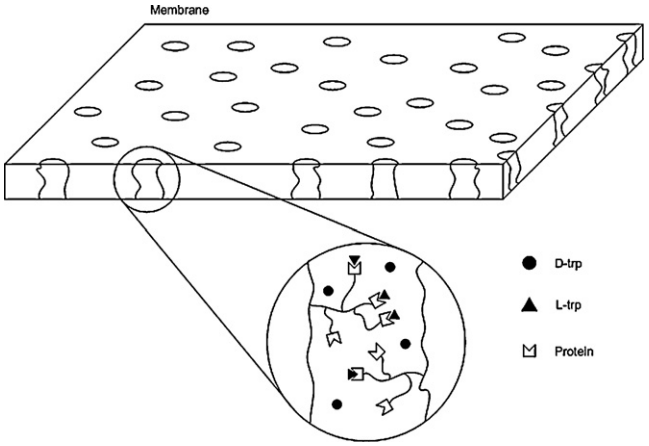

Fig. 1. The enantiomeric resolution of racemic tryptophan.

2. Materials and methods

2.1. Materials

The PVDF used in this study was Kynar HSV 900 powder supplied by Arkema Pte Ltd. NMP (>99.5%), methanol, phosphoric acid, disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) were supplied by Merck. BSA (A9418, 96+% pure), copper (I) chloride (CuCl) was obtained from Sigma; MA (95+% pure and stabilized with 1000 ppm hydroquinone) and racemic DL-tryptophan were purchased from Alfa Aesar. 2-2′ bipyridine (bpy) was obtained from Strem Chemicals. α-Cyclodextrin was obtained from Cyclo Labs. All chemicals were used as received without any further purification steps.

2.2. Synthesis of graft copolymer

CuCl was used as the catalyst in ATRP and bpy was the ligand to synthesize the PVDF–g-PMA copolymer in two different batches. Conical flasks containing 42–43 g of a 10% PVDF in NMP solution were prepared and mixed with 0.04 g of catalyst, 0.23 g of ligand and 10 ml of MA, then subjected to purging with nitrogen gas for half an hour and heated up to 90 °C for the polymerization reaction to occur for a further 18 h.

The finished product after the conclusion of ATRP was precipitated in a 1:1 (v/v) ethanol–water mixture and filtered to remove the catalyst, ligand and unreacted monomer, as well as any non-grafted PMA polymer in the polymer solution. The polymer was then re-dissolved in NMP and subjected to re-precipitation and re-dissolution in NMP until the ethanol–water filtrate from the filtration process turned colourless. The final precipitate product was washed with deionized water for 6 h to remove any traces of ethanol and dried in a Thermo ModulyoD freeze dryer (Thermo Electron Corporation, USA) overnight before being re-dissolved to form a 10 wt% polymer solution in NMP once again for casting.

The freeze-dried grafted polymer was then dissolved in NMP to make up a 10 wt% solution. The solution was spread out on a glass plate and cast with a casting knife at 250 μm thickness. The glass plate was then immersed in an ethanol bath for 20 min, followed by immersion in a tap water bath for phase separation to occur. The flat membrane films that were precipitated were then soaked in a tap water bath for 2 days with a change of water in the water bath each day, frozen, and freeze-dried overnight.

2.3. Polymer characterization via gel permeation chromatography

Dry polymer samples of PVDF powder and PVDF–g-PMA powder were dissolved to a concentration of 1 g/l in HPLC-grade dimethylformamide (DMF) and eluted with HPLC-grade DMF in a Waters GPC system equipped with a set of Waters Styragel columns, a Waters-2487 dual λ absorbance detector, and a Waters-2414 refractive index detector, calibrated against a monodisperse polystyrene standard.

2.3.1. Morphology investigation of membranes via scanning electron microscopy (SEM)

Freeze-dried flat membranes were immersed in liquid nitrogen and fractured to obtain smooth cross-sections of the membrane fibres and then sputtered with platinum using a JEOL JFC-1300 Platinum Coater at a current of 42 mA for 80 s. The SEM analysis was carried out by a JEOL JSM-5600LV Scanning Electron Microscope, and the cross-sections were analysed together with the top surfaces of the membrane fibres.

2.3.2. Membrane surface elemental composition determination via X-ray photoelectron spectroscopy (XPS)

The membrane surface compositions (both without and with adsorbed protein incubated at pH 8) were characterized by XPS, performed on a Kratos AXIS HSi spectrometer using a monochromatised Al Kα X-ray source (1486.6 eV photons) at a constant dwell time of 100 ns and a pass energy of 40 eV. The core-level signals were obtained at a photoelectron takeoff angle at 90° (with respect to the membrane surface). The X-ray penetration depth ranged between 5 and 7 nm.

2.4. Static BSA adsorption

0.1 g of these freeze-dried membrane films were washed with methanol for half an hour to pre-wet the graft copolymer surface, followed by washing with a prepared buffer solution containing 0.1 M phosphate buffer at pH 8. The membranes were then immersed in 20 ml of 1 g/l BSA solution for 24 h after washing and kept at room temperature. The BSA adsorption was calculated with a calibrated absorption curve by a UV-Vis BioChrom Libra S32 spectrophotometer at a wavelength of 280 nm by subtracting the mass of BSA present in the solution from the original mass of BSA within the solution. These samples were freeze dried and weighed to verify that the calculated BSA adsorption from the UV–vis spectrophotometer corresponded to the overall mass increase of the membrane samples. The adsorption capacity of the membrane exhibiting the highest protein adsorption data was then investigated at different pH levels, and a concentration-dependent static adsorption relationship was also drawn up for that membrane at pH 8.

2.5. Enantiomeric resolution

The membrane samples immersed in the optimal pH buffer for protein capture were washed repeatedly with pH 8 buffer to remove any desorbed protein. They were then placed in 20 ml of a 0.184 mM solution of racemic tryptophan to monitor the membrane's stereoselective efficacy under static incubation.

In addition, a permeation cell was set up and a membrane was cast upon a nonwoven fabric surface to observe the BSA's stereoselective efficacy at a given pH and given amount of tryptophan. A circular membrane disc of diameter 3.3 cm was cut out from the cast membrane, then soaked in the protein solution overnight and washed with buffer repeatedly until no further desorption of protein was recorded on the UV–vis spectrophotometer measurement. This membrane was then used in the permeation cell with 35 ml of a 0.184 mM tryptophan racemate in 0.1 M phosphate buffer at pH 8 in the feed side of the cell, and 35 ml of deionized water on the other side of the cell, with the nonwoven fabric side of the membrane in contact with the deionized water, as per similar experiments conducted by our research group previously to demonstrate the membrane's stereoselectivity [30,31]. 200 μl of permeate was extracted from the cell periodically and analysed via capillary electrophoresis (P/ACE MDQ Capillary Electrophoresis System, Beckman). The buffer used in the quantitative analysis contained 20 mM phosphate and 50 mM of α-cyclodextrin at pH 2.2. The separation factor at each individual time stage is given in Eq. (1) [25,28,29]:

$$\text{separation factor} = \frac{C_{p,D}}{C_{p,L}} \quad (1)$$

where $C_{p,D}$ and $C_{p,L}$ refer to the concentrations of D- and L-tryptophan in the permeate side, respectively. The time-averaged-separation factor can then be obtained as the ratio of the D- and L-tryptophan fluxes over the entire range of the experimental operation as a linear fit of the concentration data.

Table 1
Polymer characterization through GPC.

	M_n	M_w	PDI
Pristine PVDF	1.26×10^6	1.39×10^6	1.10
PVDF–g-PMA	1.39×10^6	1.48×10^6	1.06

3. Results and discussion

3.1. Polymer characterization by GPC

Table 1 illustrates the M_n, M_w and polydispersity index (PDI) of the PVDF polymer and the ATRP graft copolymer by GPC. The PDI of the grafted polymer was expectably found to not deviate greatly from the original PVDF polymer. A subsequent increase in M_n was noted for PVDF–g-PMA graft copolymer, and in conjunction with a negligible change in the observed PDI indicates that the control over the polymerization was reasonably good. The average chain M_n was increased by 5.94×10^4 g/mol. Given that MA's molecular weight is 70.09 g/mol, it follows that an increase in average chain M_n by 5.94×10^4 g/mol translates to an average graft chain length containing 848 units of MA per polymer chain, and the composition of the MA on the overall graft copolymer can be calculated to be 4.5 wt%.

3.1.1. Membrane characterization by XPS

Fig. 2a illustrates the presence of C=N imine bonds on the N 1s spectrum of the PVDF–g-PMA membrane with bound protein at a reference point of 284.6 eV with a peak at 398.2 eV, which is characteristically that of imine bond formation, as well as a smaller peak at 400.2 eV, representative of amide groups present on the protein. In contrast, the N 1s spectrum of the pristine PVDF membrane (Fig. 2b) exhibits a singular amide peak at 400.25 eV and it can hence be concluded that the protein on the ATRP membrane is predominantly chemically bound, while the protein on the PVDF membrane is predominantly physically bound Table 2 demonstrates that the graft copolymer membrane synthesized via ATRP possessed a oxygen content than the pristine PVDF membrane, which is indicative of a successful graft copolymerization of MA. In addition, the nitrogen content of both membranes with adsorbed protein were compared, and it is shown that the ATRP-grafted membrane was able to capture more protein than the pristine PVDF membrane. Fig. 2d also illustrates an increase in the peak height at 286.3 eV for the C 1s spectrum, which is also attributable to the C=N Schiff base formation as compared to Fig. 2c, which shows a much lower peak at 286.3 eV. Therefore, the aldehyde functionality of the grafted PMA

Table 2
Surface elemental composition analysis of the pristine PVDF membrane and the PVDF–g-PMA membrane.

	Carbon content (mol%)	Oxygen content (mol%)	Nitrogen content (mol%)
Pristine PVDF membrane	99.64	0.24	0.09
Pristine PVDF membrane with protein	85.92	8.55	5.93
ATRP membrane	96.51	3.34	0.15
ATRP membrane with protein	81.75	10.61	7.64

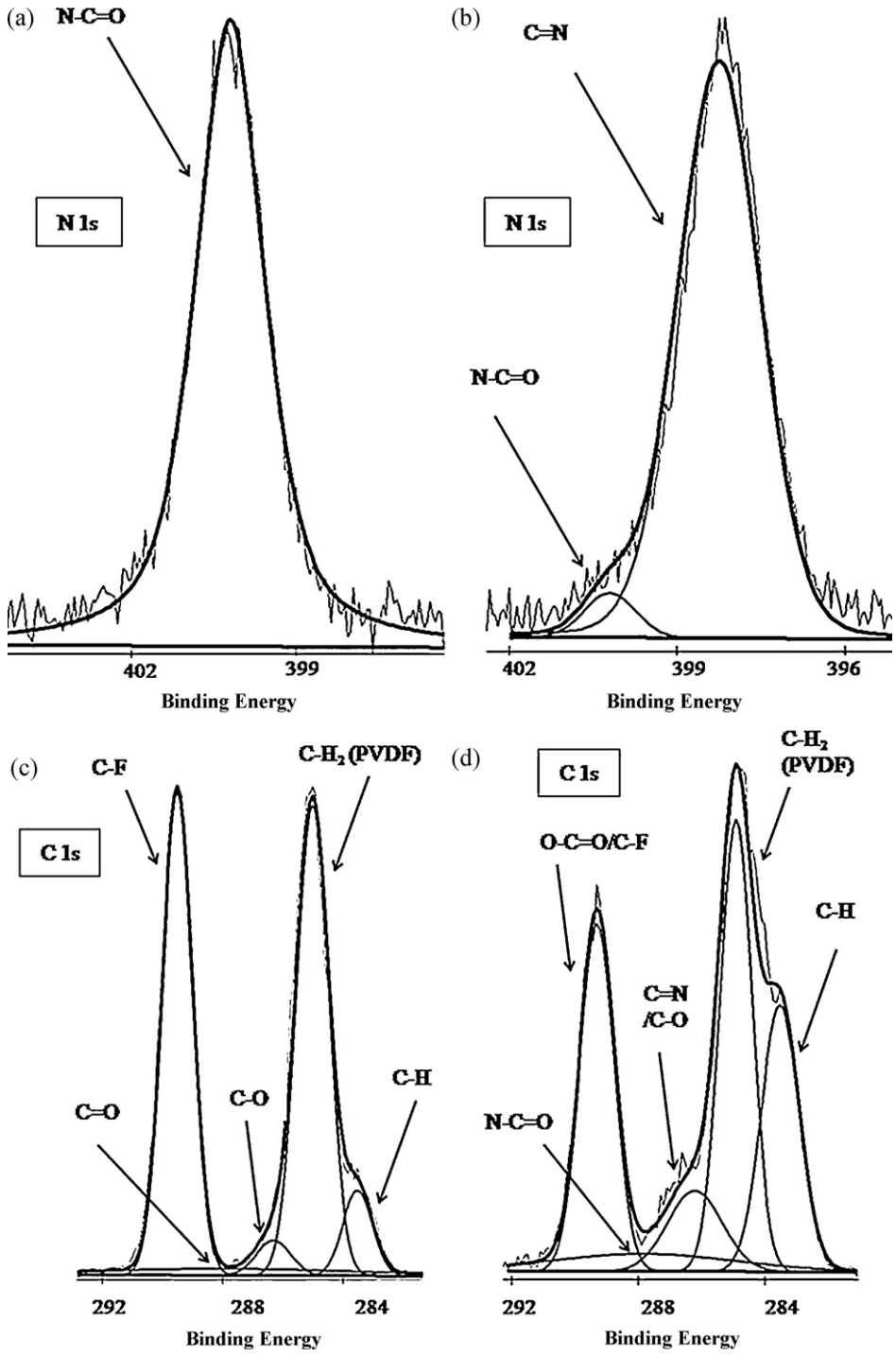

Fig. 2. (a) N 1s spectrum for the pristine PVDF membrane with bound protein, (b) N 1s spectrum for the PVDF–g-PMA membrane with bound protein, (c) C 1s spectrum for the PVDF–g-PMA without protein, (d) C 1s spectrum for the PVDF–g-PMA membrane with protein.

appears to be retained, as the nature of protein capture is found to be markedly different between the two membranes as shown in Fig. 2a and b. However, it has to be pointed out that to account for the Schiff base formation, there should theoretically be an aldehyde peak at 287.7–287.8 eV on the C 1s spectrum to account for the C=O carbonyl bonds, which is conspicuously negligible in Fig. 2c.

However, it was mentioned by Schulz previously that aldehydes are able to form aldehyde hydrate ethers upon hydration with water [32], which is shown more clearly by the peak of C–O ether bonds at 286.3 eV in comparison with the peak at 287.7 eV. Similar acetal formation can be observed when aldehydes come into contact with hydroxyl group-bearing solvents like alcohols. Schulz made a separate comment [32] that radical polymerization rarely causes the carbonyl bond to polymerize and form ether linkages; hence the ether bonds detected by XPS should be that of the aldehydes in their acetal forms. The freeze drying of membrane samples may not necessarily liberate all the water or ethanol interacting with the aldehyde groups bearing the membrane casting process, as it is essential to subject polyacrolein to pyrolytic temperatures of 200 °C to eliminate the water associated with the dihydroxytetrahydropyran rings completely [33]. The composition of free aldehydes present on the “dry” graft copolymer is therefore

Table 3
A comparison of protein adsorption at different pH levels.

BSA loading per unit mass of membrane (mg/g)	pH 2.9	pH 4.8	pH 8.0
Pristine PVDF membrane	68.5	103	83.8
Pristine PVDF membrane (after desorption)	49.9	81.9	79.4
ATRP membrane	140	164	146
ATRP membrane (after desorption)	123	151	135

extremely limited, but the formation of acetals and hemiacetals on a polyaldehyde chain is reversible and can revert back to free aldehydes which can then react with the lysine amino groups especially in the presence of H^+ ions in aqueous media; hence it can be concluded that the free aldehydes on the graft copolymer do not exist as aldehydes but as hydrates even after freeze drying.

The phenomenon of aldehyde hydrate ethers being able to participate in reactions similar to aldehydes has been noted by Schulz previously [32]. Therefore, it would be inevitable that these hydrate ethers will react with the amino lysine groups, which aids protein adsorption in conjunction with the greater surface area available for protein adsorption, and is evidenced by the imine peak in the N 1s spectrum. The covalent bonding of the protein to the polymer greatly reduces its propensity to desorb when washed due to the stability of the Schiff base linkage [2]. Therefore, the presence of the aldehyde graft branches provides a tandem effect of (i) increasing the surface area for protein adsorption as well as (ii) reducing protein desorption during the washing stage.

3.2. Static BSA adsorption

The concentration dependence of static total BSA adsorption onto the membrane can be correlated as an approximately linear relationship when low initial concentrations of 1–4 g/l BSA at pH 8 are used to study BSA loading onto the membrane. The BSA loading can thus be represented as per Eq. (2):

$$y = 25.334x + 90.772, \quad r^2 = 0.993 \tag{2a}$$

$$y = 19.243x + 89.062, \quad r^2 = 0.984 \tag{2b}$$

where Eq. (2a) represents the BSA loading before desorption and (2b) represents the loading after desorption; x denotes the initial concentration of BSA used for static incubation (g/l) and y denotes the protein loading per unit mass of membrane (mg/g membrane).

These equations demonstrate that the rate of increase of BSA adsorption (approximately 20–30 mg/g membrane) is heavily overshadowed by the rate of increase in the amount of BSA used (1 g/l). Therefore it appears so that the most efficient BSA concentration to be used among all four given BSA concentrations is 1 g/l, where the membrane is able to capture 59.8% of all available BSA protein in comparison with a mere 24.9% capture when a 4.03 g/l BSA solution is used.

The changing of pH for BSA adsorption demonstrated that the best pH for BSA adsorption occurs at its isoelectric point (p*I*) of 4.8, as shown in Table 3, which is in agreement with the data presented in Table 2. This was observed for both the pristine PVDF membrane and the PVDF–g-PMA membrane and can be explained by the fact that a protein's hydrophobicity is at its zenith when at its p*I* [34], which makes it thermodynamically more favorable for physical adsorption. In addition to the maximal physical adsorption due to the maximal protein hydrophobicity, Schiff base formation is acid-catalyzed and is optimal at a pH between 4 and 5. BSA adsorption capacity was noted to approximately double at pH 4.8 as a result of aldehyde introduction via ATRP, and the highest protein adsorption onto the membrane after 24 h was calculated to be 0.16 g BSA/g membrane for the ATRP membrane across multiple samples, representing a capture of 80% of the original protein in the solution in comparison to 0.10 g BSA/g membrane for the pristine PVDF membrane. It is interesting to note that the presence of extra H^+ ions at pH 2.8 does not aid much in enhancing the overall amount of protein captured on the membrane, suggesting therefore that the overall protein binding to the membrane comprises both physical and chemical adsorption and the total amount of protein bound is more heavily dependent on the amount of physically adsorbed protein.

MA is a hydrophobic monomer [35] and it would be expected that the PVDF–g-PMA copolymer would not be significantly hydrophilized in comparison with a pure PVDF polymer. It is said that desorption occurs more easily for a protein adsorbed onto a hydrophobic surface than to a hydrophilic surface [36]. Therefore, without any hydrophilicity enhancement to the PVDF membrane, the reduction in desorption to an almost constant amount regardless of pH is very likely attributable to the free aldehyde groups forming Schiff bases with the protein, as the Schiff bases formed are reversible but yet strong enough to prevent protein detachment when the polymer is immersed in a protein solution [2].

The repeatability of BSA adsorption from sample to sample as well as across two independently synthesized polymer batches also indicates a good uniformity in aldehyde grafting onto the polymer during graft copolymerization, though membrane washing reduced protein capture to 0.15 g BSA/g membrane, while the pristine PVDF membrane shows a greater protein desorption under the same washing conditions, and the final BSA binding for the PVDF–g-PMA membrane is roughly double that of the pristine PVDF membrane per gram of membrane used. However, protein desorption remains approximately independent of the adsorption pH for the PVDF–g-PMA membrane but the pristine PVDF membrane's protein adsorption appears to be much more dependent on pH.

3.3. SEM characterization of flat membranes

The introduction of aldehyde groups appears to have reduced the membrane cross-sectional thickness as observed via SEM imaging in Fig. 3 by more than three-fold (67 μm for the PVDF–g-PMA membrane as compared to 200 μm for the PVDF membrane). In addition, the pore size of the PVDF–g-PMA membrane appears to be smaller than that of the PVDF membrane, which also indicates a greater surface area available for physical protein adsorption. Table 3 compares the BSA loadings between the two membranes as a function of membrane volume, surface area and mass of membrane. The PVDF–g-PMA membrane has a mass-to-surface area ratio of 2.39 mg/cm^2 in comparison with 4.52 mg/cm^2 for the pristine PVDF membrane, which can be primarily due to the modification caused by the aldehyde grafting.

The protein adsorption parameters shown in Table 4 demonstrate that the BSA adsorbed by the pristine PVDF membrane appears to be higher than that of the PVDF–g-PMA membrane if a protein mass-to-surface area ratio is considered, even though the total amount of protein captured by the PVDF–g-PMA membrane is double that of the pristine PVDF membrane, due to the differences in membrane morphology as can be observed in Fig. 3.

The membrane protein binding capacity of the PVDF–g-PMA membrane can also be calculated in terms of BSA adsorbed per membrane unit volume to be 53.9 mg/cm^3 membrane given a thickness of 67 μm, or 36% of the 150 mg BSA/cm^3 membrane as presented by Sun et al. via the surface modification of a porous alumina membrane [19], but requiring only one reaction step to formulate the final membrane for protein adsorption in comparison with the four reaction steps proposed by Sun et al.

3.4. The influence of membrane pore size on enantioseparation

A comparison in pore sizes with the unmodified and modified PVDF membranes via SEM imaging shows that there is little resis-

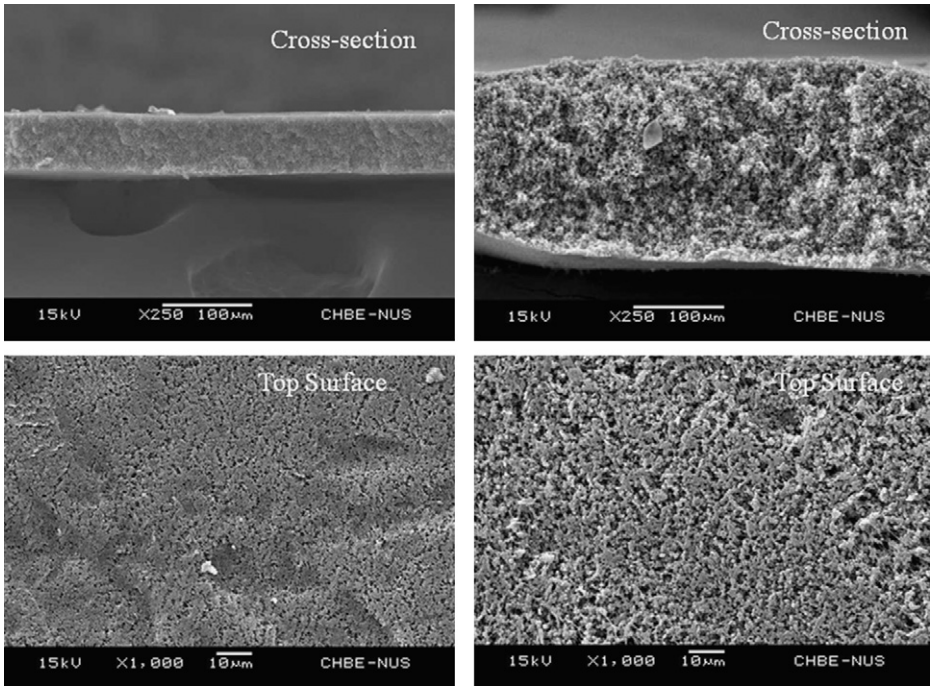

Fig. 3. Membrane cross-section and top surface SEM imaging of the PVDF–g-PMA membrane (left) and the pristine PVDF membrane (right).

Table 4
A comparison of protein adsorption between the pristine PVDF membrane and the ATRP membrane at pH 4.8.

BSA loading	Per unit area of membrane (mg/cm^2)	Per unit volume of membrane (mg/cm^3)	Per unit mass of membrane (mg/g)
Pristine PVDF membrane	0.47	28.2	103
Pristine PVDF membrane (after desorption)	0.37	18.5	81.9
ATRP membrane	0.39	58.5	164
ATRP membrane (after desorption)	0.36	53.9	151

tance to fluid flow with the formation of micron-sized pores after phase inversion in ethanol. The little resistance to fluid flow presented by the PVDF membrane as modified in solution phase shows that the grafting levels do not pose any issues of pore blockage. The tested pure water flux for the membrane was 9100 LMH at an applied pressure of 2 bar gage with negligible resistance to flow, and given the micron-sized pores of the ATRP membrane as observed in Fig. 3 and comparable with the pristine PVDF membrane, the membrane selectivity for the enantioresolution of tryptophan is not through size or charge exclusion but is solely derived from the amount of BSA immobilized onto the membrane. Tryptophan permeation across the membrane is therefore dependent on the concentration difference between the feed side and the strip side. It is expected that the permeation of the L-enantiomer is more hindered than the D-enantiomer because it is more strongly bound to BSA than the D-enantiomer is. It is highly possible that some of the tryptophan will permeate through the membrane unhindered by the bound BSA on the membrane. Tryptophan is a small amino acid of molecular weight 204.23 Da and radius of 3.3 Å [37] which can easily permeate through the micron-sized pores of the ATRP membrane without the aid of any pressure differentials and hence the stereoselectivity of the membrane will be affected.

3.5. Tryptophan stereoselectivity

Due to the linear dependence of tryptophan binding to BSA (2 mol tryptophan/mol BSA) [25], it would be assumed that the ATRP membrane would possess a better performance in the enantioresolution of tryptophan than the PVDF membrane per unit mass of membrane, other things remaining equal. Therefore, the PVDF–g-PMA membrane was incubated in 20 ml of 0.184 mM racemic tryptophan at pH 8 under static conditions to firstly verify that the BSA retained its binding ability to tryptophan, and the static incubation tryptophan binding with respect to time is shown in Fig. 4. The retention in stereospecific activity of BSA is evidenced by the different binding affinities in Fig. 4, where a greater amount of L-tryptophan is bound per mol of BSA than its corresponding

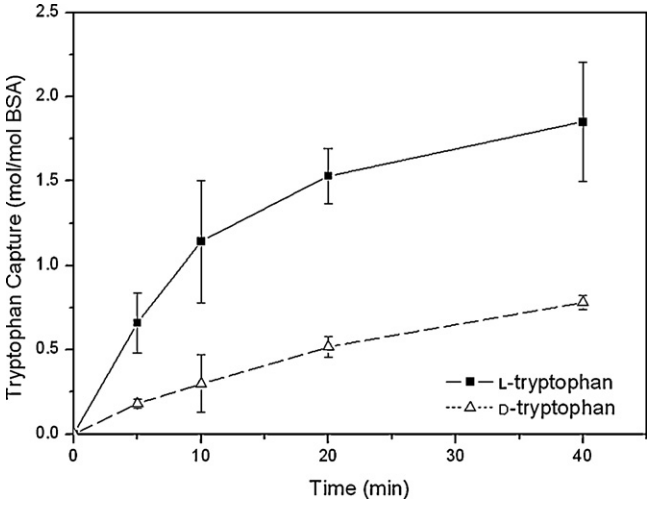

Fig. 4. Kinetics of competitive tryptophan capture in a racemic mixture.

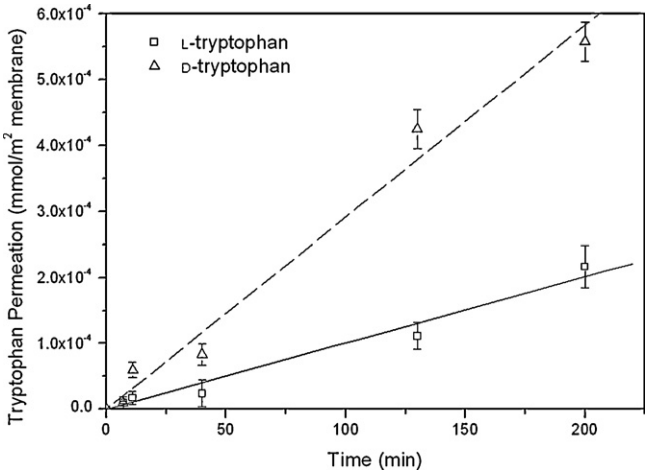

Fig. 5. Tryptophan flux in a permeation cell setup.

D-enantiomer, and was found to be 2.63 mol tryptophan/mol BSA after 40 min, which can be explained by the postulation from Lee and Frank that covalently immobilized polypeptides experience greater hydrophobic interactions with hydrophobic amino acids like tryptophan [16].

The capture of L-tryptophan was observed to be considerably faster and stronger than D-tryptophan verified across three different membrane samples, as has been detailed in previous works [25–29,34]. These results are validated by the data obtained in the permeation flux experiment (shown in Fig. 5), where it is observed that the permeation flux of the D-enantiomer is higher than that of the L-enantiomer. Tryptophan flux is known to arise from a concentration difference only and not from any size exclusion since its porosity has already been highlighted in Fig. 3 as well as the pure water flux test, leading to the conclusion that the binding sites on the BSA protein molecules assume the main responsibility for the membrane's stereoselectivity. The separation factor at each time stage based on Eq. (1) is shown in Fig. 6.

It is known that the binding site does not possess any reactive amino acid residues like lysine or cysteine [24], which possess the propensity as nucleophiles to attack the electrophilic aldehyde groups on the membrane; hence, we can make a further conclusion that the binding site structure is not significantly altered by the covalent anchoring of the protein for the retention of its binding ability.

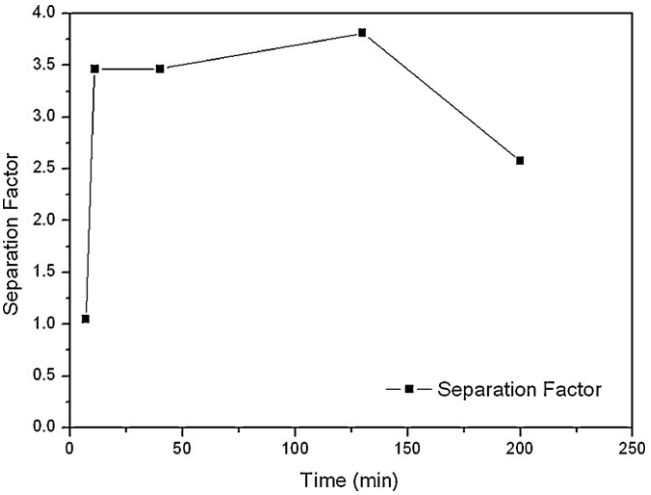

Fig. 6. Relationship of separation factor with time.

One germane observation is that the number of moles of L-tryptophan bound to the BSA exceeds the number of moles of immobilized BSA after 20 min for all three samples, which supports the purported thesis by Romero and Zydney [25] that there are two tryptophan binding sites on BSA which can bind to both L- or D-tryptophan in a competitive manner instead of there being just one stereospecific partially blocked binding site for the L-enantiomer [24]. Tryptophan capture occurs rapidly and can be seen to approach an asymptotical equilibrium after 40 min. The preferential capture of L-tryptophan by the membrane over D-tryptophan was calculated as the ratio of the number of moles of L-tryptophan bound by the membrane to the number of moles of D-tryptophan and determined to be 2.4 after 40 min.

The time-averaged permeation flux for the tryptophan in Fig. 5 was calculated to be 2.9. Previous ultrafiltration experiments with free BSA and racemic tryptophan have shown a separation factor of 5 for a BSA concentration of 10.1 g/l and a tryptophan concentration of 0.05 mM at pH 7 with polyacrylonitrile hollow fibre membranes [28], while other experiments have obtained a separation factor in excess of 6 with a 21 g/l BSA concentration and a 0.2 mM tryptophan concentration with polyethersulfone (PES) membranes at pH 8.5 [25]. A parallel study in our research group utilizing a 13 g/l BSA solution and 0.1 mM tryptophan at pH 8 with PES membranes yielded a separation factor of 1.6 [29].

However, here in this study we have immobilized BSA onto a porous membrane support and achieved a time-averaged-separation factor of 2.9 in the separation of a 0.184 mM tryptophan solution with the initial use of just 20 ml of a 1 g/l BSA solution, which is highly remarkable because it has reduced the requirement for the protein macroligand tremendously. Nevertheless, the time dependency of the separation factor as depicted in Fig. 6 shows a decrease in the membrane's effective separation factor as the BSA molecules become saturated with tryptophan molecules over time. This is inevitable if the bound tryptophan is not released by the BSA molecules quickly enough into solution, as it has been established that there is negligible diffusion resistance of L-tryptophan to BSA [10] and may be a drawback to the entire enantioseparation process especially if conducted over a long period of time.

4. Perspectives and conclusions

We have demonstrated the ability of a facile single-step ATRP synthesis here in this study to develop a PVDF–g-PMA copolymer membrane material that is able to double its protein binding capacity with a good control over chain length and polydispersity, with a good ability to immobilize proteins primarily via covalent Schiff base linkages. The protein used in immobilization was further found to retain its stereospecific activity in the enantioresolution of racemic tryptophan, with a total tryptophan binding per molecule of BSA that parallels the hypothesis put forth by Romero and Zydney [25]. The total equilibrium binding of both tryptophan enantiomers was found to exceed 2 mol/mol BSA as seen in Fig. 6, which is indicative that both the primary and secondary binding sites on the BSA protein may be unaffected by the covalent immobilization. However, the fact that some tryptophan molecules may be physically adsorbed onto the membrane surface or hydrophobically attracted to the covalently bound BSA [16] non-specifically has to be taken into account as well, as this non-specific permeation-retarded molecular transport of tryptophan will intrinsically factor into the determination of the membrane selectivity. Though the overall physical structure of BSA may be altered when it is immobilized on a support layer [2], the binding site of the protein is the major determining factor of the membrane's selectivity and the retention of its activity is of utmost importance.

It is interesting to notice that the grafted aldehydes on the membrane do not exist as free aldehydes but as a tetrahydropyranose structure, which when contacted with water after pre-wetting with methanol is able to revert back into the aldehyde structure for Schiff base reaction and formation. This unique behavior of hydrated aldehydes protects the aldehydes from being easily degraded by atmospheric oxygen, and can preserve the PVDF–g-PMA's aldehyde functionality significantly. The newfound ease of covalent protein immobilization onto the PVDF membrane backbone through this synthesis method opens up an avenue for the development of new enzymatic membrane reactors for the combination of biocatalytic reactions with filtration processes in a single reaction vessel. Though the protein loading of such membranes may not be as high as other conventional membrane adsorbers, it must be noted that the synthesis method is one of the most convenient methods to be used.

It is known that the protein capture is dependent on the grafting degree of the aldehydes onto the polymer, and grafting degree is in turn dependent on the polymerization reaction conditions like reaction duration, monomer concentration, choice of catalyst and activator–deactivator ratio [38]. Therefore, it can be further postulated that membrane selectivity is a controllable parameter, and the ATRP reaction conditions can therefore be further optimized to increase the selectivity of the entire process, especially given the low weight composition of poly(MA) in the PVDF copolymer at 4.5 wt%. The high porosity of the cast membrane might also result in some of the tryptophan molecules not interacting with the BSA and permeating unhindered through the membrane, which also results in a loss of selectivity; hence it can be recommended that the final dope used for casting should incorporate a higher composition of graft copolymer.

We caution that this membrane be used carefully as the immobilization results might reduce the activity of different enzymes. In the case of BSA used in this study, the binding site of the BSA does not contain reactive functional groups which can bind covalently to the aldehyde molecules [24], hence its stereoselective performance is not greatly affected by the Schiff base linkages. However, the immobilization of other enzymes containing lysine and cysteine within or near their active sites may face a more severe conformational change in the binding site, which affects the enzyme molecule's biocatalytic activity.

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References

[1] H.A. Weetall, Anal. Chem. 46 (1974) 602A.
[2] T. Basinska, D. Kowalczyk, B. Miksa, S. Slomkowski, Polym. Adv. Technol. 6 (1995) 526.
[3] S. Slomkowski, Prog. Polym. Sci. 23 (1998) 815.
[4] S. Slomkowski, D. Kowalczyk, M.M. Chehimi, M. Dealamar, Colloid Polym. Sci. 278 (2000) 878.
[5] R. Sternberg, D.S. Bindra, G.S. Wilson, D.R. Thévenot, Anal. Chem. 60 (1988) 2781.
[6] T. Hayashi, Y. Ikada, Biotechnol. Bioeng. 35 (1990) 518.
[7] A. Higuchi, Y. Ishida, T. Nakagawa, Desalination 90 (1993) 127.
[8] M. Nakamura, S. Kiyohara, K. Saito, K. Sugita, T. Sugo, J. Chromatogr. A 822 (1998) 53.
[9] M. Nakamura, S. Kiyohara, K. Saito, K. Sugita, T. Sugo, Anal. Chem. 71 (1999) 1323.
[10] S. Kiyohara, M. Nakamura, K. Saito, K. Sugita, T. Sugo, J. Membr. Sci. 152 (1999) 143.
[11] V. Smuleac, D.A. Butterfield, D. Bhattacharyya, Langmuir 22 (2006) 10118.
[12] A.J. Guiomar, T. Guthrie, S.D. Evans, Langmuir 15 (1999) 1198.
[13] T. Kawai, H. Kawakita, K. Sugita, K. Saito, M. Tamada, T. Sugo, H. Kawamoto, J. Agric. Food Chem. 50 (2002) 1073.
[14] H.H. Rmaile, J.B. Schlenoff, J. Am. Chem. Soc. 125 (2003) 6602.
[15] T.N. Warner, S. Nochumson, Mod. Drug Discov. 6 (2003) 45.
[16] N.H. Lee, C.W. Frank, Polymer 43 (2002) 6255.
[17] E.T. Kang, Y. Zhang, Adv. Mater. 12 (2000) 1481.
[18] Y. Chen, D. Liu, Q. Deng, X. He, X.J. Wang, Polym. Sci.: Part A: Polym. Chem. 244 (2006) 3434.
[19] L. Sun, J. Dai, G.L. Baker, M.L. Bruening, Chem. Mater. 18 (2006) 4033.
[20] J.F. Hester, P. Banerjee, Y.-Y. Won, A. Akthakul, M.H. Acar, A.M. Mayes, Macromolecules 35 (2002) 7652.
[21] J. Xue, L. Chen, H.L. Wang, Z.B. Zhang, X.L. Zhu, E.T. Kang, K.G. Neoh, Langmuir 24 (2008) 14151.
[22] Y.-C. Chiang, Y. Chang, A. Higuchi, W.-Y. Chen, R.-C. Ruaan, J. Membr. Sci. 339 (2009) 151.
[23] S. Samanta, D.P. Chatterjee, S. Manna, A. Mandal, A. Garai, A.K. Nandi, Macromolecules 42 (2009) 3112.
[24] R.H. McMenamy, J.L. Oncley, J. Biol. Chem. 233 (1958) 1436.
[25] J. Romero, A.L. Zydney, J. Membr. Sci. 209 (2002) 107.
[26] F. Garnier, J. Randon, J.L. Rocca, Sep. Purif. Technol. 16 (1999) 243.
[27] V. Tittelbach, R.K. Gilpin, Anal. Chem. 67 (1995) 44.
[28] E. Iritani, N. Katagiri, T. Kawabata, Y. Takaishi, Sep. Purif. Technol. 64 (2009) 337.
[29] H. Wang, Y. Li, T.-S. Chung, AIChE J. 55 (2009) 2284.
[30] Y. Xiao, H.M. Lim, T.-S. Chung, R. Rajagopalan, Langmuir 23 (2007) 12990.
[31] Z. Zhou, Y. Xiao, T.A. Hatton, T.-S. Chung, J. Membr. Sci. 339 (2009) 31.
[32] R.C. Schulz, Angew. Chem. Int. Ed. 3 (1964) 416.
[33] L. Hunter, J.W. Forbes, J. Polym. Sci.: Part A 3 (1965) 3471.
[34] P.-C. Wang, J. Gao, C.S. Lee, J. Chromatogr. A 942 (1999) 115.
[35] W. Albrecht, J. Schauer, Th. Weigel, A. Lendlein, J. Membr. Sci. 269 (2006) 49.
[36] Y.L. Jeyachandran, E. Mielczarski, B. Rai, J.A. Mielczarski, Langmuir 25 (2009) 11614.
[37] K. Willaert, Y. Engelborghs, Eur. Biophys. J. 20 (1991) 177.
[38] J. Xia, K. Matyjaszewski, Chem. Rev. 101 (2001) 2921.