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An all-aqueous route to polymer brush-modified membranes with remarkable permeabilities and protein capture rates

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Abstract

Microporous membranes are attractive for protein purification because convection rapidly brings proteins to binding sites. However, the low binding capacity of such membranes limits their applications. This work reports a rapid, aqueous procedure to create highly permeable, polymer brush-modified membranes that bind large amounts of protein. The synthetic method includes a 10-min adsorption of a macroinitiator in a hydroxylated nylon membrane and a subsequent 5-min aqueous atom transfer radical polymerization of 2-(methacryloyloxy)ethyl succinate from the immobilized initiator to form poly(acid) brushes. This procedure likely leads to more swollen, less dense brushes than polymerization from silane initiators, and thus requires less polymer to achieve the same binding capacity. The hydraulic permeability of the poly(acid) membranes is 4-fold higher than that of similar membranes prepared by growing brushes from immobilized silane initiators. These brush-containing nylon membranes bind 120 mg/cm³ of lysozyme using solution residence times as short as 35 ms, and when functionalized with nitrilotriacetate (NTA)-Ni²⁺ complexes, they capture 85 mg/cm³ of histidine₆-tagged (His-tagged) Ubiquitin. Additionally the NTA-Ni²⁺-functionalized membranes isolate His-tagged *myo*-inositol-1-phosphate synthase directly from cell extracts and show >90% recovery of His-tagged proteins.

Keywords

Affinity membranes; protein purification; macroinitiator; His-tagged protein; polymer brushes

1. Introduction

Although rapid advances in biotechnology have greatly increased the synthesis and applications of recombinant proteins, isolation of these proteins remains a bottleneck in their production [1, 2]. Specific adsorption of proteins to ligands immobilized in packed columns is probably the most powerful step in protein purification [3, 4]. Nevertheless, high pressure drops, challenges in packing large columns and slow mass transfer may limit the utility of column-based separations [4–6]. Membrane adsorbers [7] provide an alternative for rapid protein isolation because flow through functionalized membrane pores rapidly transports the protein of interest to immobilized ligands. Moreover, scale-up of membrane separations by

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increasing surface area is relatively simple and causes little or no increase in pressure drop [6, 8–15].

Despite the possibility of fast separations, however, membranes suffer from a modest binding capacity due to their low internal surface area relative to nanoporous beads [16]. In attempts to increase capacity, a number of groups grafted functionalized polymer chains to the pores of the membrane. The chains contain multiple binding sites, and membranes modified with polymer brushes show high binding capacities and selective protein binding [5, 11, 17–26]. For example, Yang and Ulbricht grafted poly(2-hydroxyethyl methacrylate) (poly(HEMA)) in poly(ethylene terephthalate) track etched membranes and then grew poly(2-lactobionamidoethyl methacrylate) from poly(HEMA) to form a well-defined comb-like glycopolymer layer. Such membranes capture as much as 24 mg/cm³ of lectins that specifically bind to galactose [19]. Bhut and Husson grafted poly(2-dimethylaminoethyl methacrylate) brushes in regenerated cellulose to form anion-exchange membranes with protein binding capacities of 130 mg/cm³ [5]. These membranes successfully captured anthrax protective antigen protein from *E-coli* cell extracts [27].

The most common method for specific protein isolation exploits interactions between polyhistidine-tagged (His-tagged) protein and metal-ion complexes. Several recent studies demonstrate specific binding of His-tagged proteins to metal-ion complexes in polymer brushes [11, 18, 20–22, 28]. We showed that growth of poly(2-(methacryloyloxy)ethyl succinate (poly(MES)) brushes in nylon membranes and functionalization of the brushes with nitrilotriacetate (NTA)-Ni²⁺ complexes leads to membranes that isolate His-tagged cellular retinaldehyde binding protein directly from cell extracts [21]. Nevertheless, modification of polymeric membranes is challenging because the membranes often dissolve or swell in organic solvents.

This work describes a simple, rapid, and completely aqueous procedure for growth and functionalization of polymer brushes in nylon, polyethersulfone (PES), and polyvinylidene fluoride (PVDF) membranes. The approach combines adsorption of a macroinitiator, atom transfer radical polymerization (ATRP) of MES (a water-soluble, acid-containing monomer) from the initiator, and subsequent aqueous derivatization. Although we demonstrated these different steps previously [21, 29], this is the first application of macroinitiator adsorption to create protein-adsorbing membranes. Most importantly, the brushes grown from these initiators behave very differently from brushes grown from silane-based initiators immobilized in membranes, presumably because of a lower density of grafted polymer chains. Compared to grafting using a trichlorosilane initiator, the macroinitiator-modified membranes require much shorter polymerization times (5 min versus 1 h) to achieve similar protein-binding capacities. Moreover, when using the macroinitiators, modified polymer membranes have 4-fold less hydraulic resistance than membranes prepared with the trichlorosilane initiator. Thus, these new systems are attractive for rapid purification of His-tagged protein directly from cell extracts. Remarkably, lysozyme capture in these membranes can occur during a ~35 ms residence time.

2. Experimental

2.1. Materials

Hydroxylated nylon (LoProdyne® LP, Pall, 1.2 µm pore size, 110 µm thick), nylon (GE, non-hydroxylated, 1.2 µm pore size, average thickness 95 µm), polyethersulfone, (GE, 1.2 µm pore size, average thickness 130 µm), hydrophilic PVDF (Millipore, 0.45 µm pore size, 115 µm thick), and regenerated cellulose membranes (Whatman, RC 60 – 1 µm pore size) were cut into 25 mm-diameter discs prior to use. Coomassie protein assay reagent (Thermo Scientific), Histidine₆-tagged Ubiquitin (HisU) (human recombinant, Enzo Life Sciences),

Concanavalin A from *Canavalia ensiformis* (Jack bean) Type IV (Con-A, Sigma Aldrich), tris[2-(dimethylamino)ethyl]amine (Me₆(TREN), ATRP Solutions), and other chemicals from Sigma-Aldrich were used as received unless noted otherwise. Trichlorosilane initiator (11-(2-bromo-2-methyl)propionyloxy)-undecyltrichlorosilane [30], and the macroinitiator (poly(2-(trimethylammonium iodide)ethyl methacrylate-co-2-(2-bromoisobutyroxy)ethyl acrylate)) [29, 31] were synthesized according to literature procedures. Buffers were prepared using analytical grade chemicals and deionized (Milli-Q, 18.2 MΩ cm) water.

2.2. Initiator attachment

Membranes discs were cleaned for 10 min with UV/ozone, and placed in a homemade teflon cell that exposed an external membrane surface area of 3.14 cm². (In a few cases we used larger membranes and holders that exposed 11.4 cm² external surface area.) The macroinitiator solution (2 mg/mL in water) was then passed through the membrane for 10 min at ~1 mL/min using a peristaltic pump, and the membrane was rinsed with 20 mL of water at the same flow rate before drying in a stream of N₂. For membranes other than hydroxylated nylon, prior to adsorption of the macroinitiator a polystyrene sulfonate (PSS) (*M_w* ~70 000) layer was deposited by passing 10 mL of 0.02 M aqueous PSS (containing 0.5 M NaCl) through the membrane at 1 mL/min. Water (10 mL) was pumped through the membrane after deposition of PSS, followed by the macroinitiator and a subsequent 10 mL water rinse. Trichlorosilane initiator attachment occurred by circulating a 1 mM initiator solution in 20 mL of anhydrous THF through the clean nylon membrane for 2 h at a flow rate of 3 mL/min, followed by subsequent rinsing with 20 mL THF and 20 mL of ethanol. The membrane was dried under a steam of N₂ prior to polymerization.

2.3. Polymer brush synthesis

Using our prior procedure [21, 32], poly(MES) brushes were grown from membranes coated with initiators. A 10 mL mixture of neat MES monomer and 1 M aqueous NaOH (1:1, v/v) was degassed with three freeze-pump-thaw cycles. A 1 mL solution of anhydrous dimethyl formamide (DMF) containing CuBr (2 mM), CuBr₂ (1 mM), and Me₆(TREN) (6 mM) was similarly degassed, and in a N₂-filled glove bag, this solution of catalyst was mixed with the monomer/NaOH solution. Polymerization of MES within the pores of the membranes (Figure 1) occurred in a N₂-filled glove bag by circulating the polymerization/catalyst solution through the initiator-modified membrane at a flow rate of 1 mL/min. *Unless mentioned otherwise, the polymerization time for membranes was 5 min.* Immediately after polymerization, 20 mL of ethanol followed by deionized water (20 mL) were passed through the membrane at ~2 mL/min. In the case of polymeric membranes other than hydroxylated nylon, the catalyst salts and ligand were added directly to the monomer-solvent mixture during the degassing process to avoid the presence of DMF, which may dissolve membranes like PES. (Direct addition of catalyst to the monomer solution is generally preferable, but we used the DMF catalyst solution with hydroxylated nylon membranes to facilitate comparisons with our prior results [21].) Functionalization of the poly(MES) side chains with NTA-metal ion complexes occurred as reported previously (Figure 1) [21, 32]. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Perkin Elmer Spectrum One Instrument, air background) as well as field-emission scanning electron microscopy (FESEM, Hitachi S-4700II) verified film growth on polymer membranes.

2.4. Protein binding

Unless specified otherwise, all protein-binding studies employed hydroxylated nylon substrates. A solution of lysozyme (0.30 or 1.0 mg/mL) in 20 mM phosphate buffer (pH 7.4) was pumped through the poly(MES)-modified membrane using a peristaltic pump (flow rate of 1 or 30 mL/min), and the permeate was collected for analysis at specific time intervals.

Breakthrough curves show the protein concentration plotted at the end of each of these intervals. Subsequently, the membrane was rinsed with 20 mL of washing buffer I (20 mM phosphate buffer with 0.1 % Tween 20, pH 7.4) followed by 20 mL of phosphate buffer. The protein was then eluted using 5–10 mL of 20 mM phosphate buffer (pH 7.4) containing 1 M KSCN.

Similar procedures with different buffers and eluents were used for Con A and HisU binding studies. In the case of HisU, the washing buffer (washing buffer II, 20 mM phosphate buffer containing 0.1% Tween-20 surfactant and 0.15 M NaCl) and elution buffer (0.5 M NaCl and 0.5 M imidazole in 20 mM phosphate buffer) were maintained at pH 7.4. For Con A, a solution of 0.30 or 0.10 mg protein/mL was made in 20 mM phosphate buffer of pH 6 to avoid protein aggregation. The washing buffer II and elution buffer (20 mM phosphate buffer containing 50 mM EDTA) were also maintained at pH 6. For Con A binding, the membrane could be reloaded with Cu^{2+} by passing a 0.1 M CuSO_4 solution through the membrane and rinsing prior to reuse. The concentrations of protein in loading, rinsing, and eluate solutions were determined using a Bradford assay as described in supplementary material. In each case, 3 membranes were individually tested, and the average binding capacity is reported. (For HisU only 2 measurements were performed due to the high cost of the protein.) Error bars and reported uncertainties are standard deviations.

2.5. Protein separation from a cell extract and determination of protein purity

His-tagged *myo*-inositol-1-phosphate synthase (MIPS) was over-expressed in *E.coli* cells. The cells were lysed with sonication in 20 mM, pH 8 phosphate buffer that contained 10 mM imidazole and 300 mM NaCl, and centrifuged at 4 °C. Supernatant (5 mL) was pumped thorough the poly(MES)-NTA- Ni^{2+} -modified nylon membrane at room temperature at a flow rate of 1.5 mL/min. Subsequently the membrane was rinsed with 20 mL washing buffer II at pH 8 followed by 20 mL washing buffer III (pH 8, 20 mM phosphate buffer with 45 mM imidazole and 0.15 M NaCl), and the bound protein was eluted with 10 mL (2 mL fractions) of 20 mM, pH 8 phosphate buffer with 0.5 M NaCl and 0.5 M imidazole. The purity of the eluted protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 4–20% gradient gel obtained from Bio-Rad was used, and the protein bands were visualized using standard silver staining [33] or Coomassie blue staining protocols [34].

3. Results and Discussion

3.1. Synthesis and characterization of polymer brushes in hydroxylated nylon membranes

To create initiation sites for polymerization in membranes, we simply pass a macroinitiator solution through the unmodified membrane and rinse the system with water. Hydrophobic interactions between the backbones of the macroinitiator and the nylon membrane presumably provide strong adsorption of the macroinitiator. After a subsequent 5-min polymerization by pumping a MES/catalyst solution through the membrane, ATR-IR spectra clearly show a carbonyl peak at 1740 cm^{-1} (see Figure S-2, figure numbers beginning with S refer to the supplementary material), consistent with the presence of poly(MES) in the membrane. Remarkably, the macroinitiator adsorption is sufficiently robust to allow reuse of brush-modified membranes at least 5 times for lysozyme adsorption (Figure S-3). Activation of the carboxylic acid groups of poly(MES) followed by reaction with aminobutyl NTA and complexation of a metal ion (Figure 1) lead to polymer brushes containing immobilized metal ion complexes capable of binding protein. The supplementary material describes characterization of the derivatization process using ATR-IR spectroscopy (Figure S-2). SEM images also provide evidence for the polymerization and derivatization (Figure S-4).

Because the amount of polymer in the membranes is low and ATR-IR spectra are qualitative, monitoring the growth of poly(MES) in the membrane is difficult. We can, however, readily determine the amount of Cu^{2+} that binds to membranes modified with poly(MES)-NTA brushes. Figure 2 shows that the amount of Cu^{2+} captured in these membranes increases rapidly with the time allotted for polymerization of MES from the macroinitiator. However, after 30 min of polymerization, the Cu^{2+} -binding capacity reaches a plateau at 25 mg of Cu^{2+} per cm^3 of membrane. The initial increase reflects growth of polymer repeat units in the membrane, whereas the subsequent plateau may stem from termination of polymerization or steric hindrance to Cu^{2+} binding. The relatively rapid ATRP in this system may lead to significant termination and some dispersion in chain molecular weights. However, low polydispersity is likely not a requirement for extensive and rapid protein binding.

As we show in section 3.3, 5 min of MES polymerization is sufficient to achieve maximum protein binding. After modification with NTA, the membranes modified with a 5-min polymerization time bind 10 ± 0.4 mg of Cu^{2+} per cm^3 of membrane. Assuming every NTA binds one Cu^{2+} ion and every carboxylic acid group of poly(MES) reacts with one aminobutyl NTA molecule, this amount of Cu^{2+} implies 35 mg of poly(MES) (before derivatization) per cm^3 of the membrane (the membrane volume, 0.035 cm^3 includes both porous and nonporous regions). For comparison, maximum protein binding when growing poly(MES) brushes from trichlorosilane initiator-modified nylon membranes requires 1 h of polymerization, and Cu^{2+} analysis suggests that this system contains 110 mg poly(MES)/ cm^3 of membrane [21].

3.2. Membrane Permeability

Effective membrane adsorbers should have both a high binding capacity and a reasonably high permeability. The experimental procedure to measure the hydraulic permeability through the membrane is described in the supplementary material along with the apparatus (Figure S-1). At 0.68 atm, a bare hydroxylated nylon membrane with a $1.2 \mu\text{m}$ nominal pore size has a pure water hydraulic permeability of $118 \pm 1 \text{ mL}/(\text{cm}^2 \text{ min atm})$, but this value drops to $85 \pm 4 \text{ mL}/(\text{cm}^2 \text{ min atm})$ after adsorption of the macroinitiator and a 5-min polymerization of MES. The polymer brushes presumably decrease pore diameters and reduce permeability. Because permeability is proportional to pore diameter raised to the fourth power, even thin films cause a significant drop in flux. After immobilization of aminobutyl NTA, the hydraulic water permeability decreases to $69 \pm 4 \text{ mL}/(\text{cm}^2 \text{ min atm})$ due to the increase in brush thickness. Complexation of Cu^{2+} or Ni^{2+} by NTA does not significantly alter the permeability ($76 \pm 6 \text{ mL}/(\text{cm}^2 \text{ min atm})$). Notably, however, even after growth of poly(MES) for 5 min, derivatization with aminobutyl NTA, and formation of the NTA-metal ion complex, the pure water flux only decreases by 35%.

For hydroxylated nylon membranes modified with poly(MES) brushes grown from trichlorosilane initiators, the water permeability is only $21 \pm 4 \text{ mL}/(\text{cm}^2 \text{ min atm})$, $1/4$ of that for the macroinitiator-based modification [21]. The trichlorosilane-based system requires longer polymerization times (1 h versus 5 min) to achieve binding capacities similar to those of the macroinitiator-based membranes, and the longer time results in more polymerization and smaller pore diameters. After derivatization with NTA-metal ion complexes, the trichlorosilane-based modification still gives 4-fold lower flux than growth of brushes from the macroinitiator and subsequent derivatization.

Figure 3 shows water permeability as a function of the time allowed for MES polymerization from adsorbed macroinitiators. The permeability decreases continuously with polymerization time, and after 30–45 min of polymerization, the macroinitiator-based membranes have a permeability similar to membranes modified with the trichlorosilane

initiator and a 1-h polymerization. This suggests that 30–45 min of polymerization from the macroinitiator gives the same amount of poly(MES) in the membrane as the 1-h polymerization from the trichlorosilane initiator, which is consistent with Cu^{2+} binding capacities. A 30 min polymerization from the macroinitiator (followed by derivatization with aminobutyl NTA) leads to binding of 27 ± 2 mg of Cu^{2+} per cm^3 of membrane, whereas 1 h polymerization from immobilized trichlorosilane initiators gives 29 ± 4 mg/ cm^3 [21]. We speculate that the poly(MES) chains are more separated and swollen when grown from the macroinitiator (at least initially), so binding a given amount of protein requires less polymer brush, which leads to a lower hydraulic resistance. Thus, a 5-min polymerization is sufficient to achieve high protein-binding capacities when polymerizing from the macroinitiator even though only a small amount of polymer is present in the membrane.

Because protein binding typically occurs in buffers and not pure water, we also examined the permeability of membranes in pH 7.4, 20 mM phosphate buffer. Deprotonation of the acid groups of polymer brushes in buffers can effect a dramatic increase in brush swelling and decrease flux [35–37]. At 0.68 atm, the macroinitiator-based, poly(MES)-modified nylon membrane exhibits a buffer permeability of 37 ± 3 mL/(cm^2 min atm), about 2 times less than the initial pure water permeability. However, after passing buffer through the membrane, the pure water permeability drops to 18 ± 3 mL/(cm^2 min atm). In subsequent measurements, the buffer permeability is still 37 ± 3 mL/(cm^2 min atm) and the pure water permeability remains 18 ± 3 mL/(cm^2 min atm). After synthesis, pure water may not be sufficient to deprotonate the brush, so the water flux is higher than the buffer flux. In contrast, after deprotonation in buffer, the brush likely remains ionized in pure water so hydraulic permeability decreases. Compared to pure water, the high ionic strength (50 mM) of the buffer collapses deprotonated brushes somewhat, so flux is higher in the buffer than pure water once deprotonation occurs [35]. Relative to the buffer solution, flux increases 1.5-fold in 0.1 M NaCl, further indicating that the brushes collapse at high ionic strength to enhance permeability. The buffer flux through trichlorosilane-based poly(MES) modified membranes (8 ± 3 mL/(cm^2 min atm)) is 4- to 5-fold less than that through macroinitiator based poly(MES) modified membranes.

3.3. Lysozyme Binding

Lysozyme binds to poly(MES) by ion exchange and provides a convenient, inexpensive probe of how protein binding varies with polymerization time in poly(MES)-modified membranes. Figure 3 shows lysozyme binding determined from breakthrough curves for a series of membranes prepared using different MES polymerization times. The lysozyme binding increases when going from a 2-min to a 5-min polymerization, presumably because of more binding sites in thicker brushes. However, with further polymerization the brushes likely become more crowded so the interior adsorption sites are less accessible to proteins, and binding does not increase. Longer brushes may even block protein access to relatively small pores and decrease binding. The trend in Figure 3 is very different from that for the amount of Cu^{2+} bound by poly(MES)-NTA (Figure 2) because Cu^{2+} is much smaller than proteins.

Nevertheless, binding 113 ± 8 mg lysozyme/ cm^3 of membrane with only 5 min of polymerization is comparable to 1 h polymerization using a similar membrane and a trichlorosilane initiator (118 ± 8 mg/ cm^3) [21]. Remarkably, the macroinitiator method allows membrane modification in much less time (10 min as opposed to 2 h for initiator attachment prior to rinsing, and 5 min as opposed to 1 h for polymerization) than the trichlorosilane initiator method and gives the same binding and a 4-fold greater permeability. The binding determined from lysozyme elution from the poly(MES)-modified membrane (5-min polymerization from the macroinitiator) is 108 ± 13 mg/ cm^3 , comparable to that measured from breakthrough curves.

We note that the binding amounts reported in this section resulted from passing only 15 mL of 0.3 mg/mL lysozyme solution through the membrane, which is not sufficient to completely fill all binding sites. We maintained this procedure to compare Figure 3 with similar data reported for the trichlorosilane system [21]. Figure S-5 shows the breakthrough curves for the macroinitiator-based system. In the remainder of the manuscript, binding capacities are those at saturation, unless mentioned otherwise.

3.4. Protein binding capacity as a function of flow rate

Membrane adsorbers are particularly attractive for rapid separations because radial diffusion distances are short, and convection brings proteins to binding sites. Moreover, rapid flow rates can occur with only modest pressure drops [13, 14]. We compared the breakthrough curves for lysozyme binding to poly(MES)-modified membranes at flow rates of 1 and 30 mL/min. These flow rates correspond to linear velocities of 19 cm/h and 570 cm/h, and residence times of ~1000 msec and 35 msec, respectively. (Note that these residence times assume a membrane porosity of 50%, whereas the linear velocity is that above the membrane.) As Figure 4 shows, the breakthrough curves are essentially independent of the flow rate over this range, so a 30-fold increase in linear velocity does not lead to early breakthrough and a change in the dynamic binding capacity. Thus, neither diffusion nor binding kinetics limit protein binding at these linear velocities, and adsorption occurs in less than 35 msec. The membrane geometry likely determines the shape of the breakthrough curves, as breakthrough will occur sooner for large pores than smaller ones.

Recommended linear velocities through typical commercial gels are 150 cm/h [38] (compared to 570 cm/h for membranes), and the binding capacity for His-tagged proteins in such resin-packed columns is about 2-fold less than the membranes in this study (40–50 mg/mL of His-tagged protein compared to 85 mg/cm³ in the membrane) [38]. Thus the brush-modified membranes have the potential to greatly increase the rate of protein binding.

3.5. Scaling up poly(MES)-modified membranes

The high permeability of membranes modified using macroinitiators should permit the use of membrane stacks to increase binding capacity without creating high pressure drops [39]. To test this concept, we polymerized MES for 5 min in 5 different membranes and stacked these membranes in a holder. The pure water flux through this stack was 5 ± 0.06 mL/(cm² min atm), which is ~20-fold less than the flux through a single membrane (85 ± 4 mL/(cm² min atm)). The flux was ¼ of the expected value, possibly because misalignment between pores in adjacent spongy membranes leads to high pressure drops between membranes.

The lysozyme binding capacity of the membrane stack was 76 ± 8 mg/cm³ or about 30 % less than individual membranes. This also might result from blocked inlets or outlets of some pores due to stacking. Nevertheless, the binding capacity of the stack of 5 membranes is still 3.5-fold more than the capacity of a single membrane, and the linear velocity through the membrane (~175 cm/hr at 0.68 atm) is comparable to the recommended flow rate for commercial columns. Placing small spacers between the membranes might increase both binding capacity and permeability.

We produced a stack of 5 membranes in two different ways. In one case, we modified the membranes individually prior to stacking, and in the second variation, we stacked 5 membranes in the holder prior to modification. Both methods yield comparable binding and permeability, suggesting that simultaneous modification of many membranes can occur without a loss of polymerization efficiency.

Scale up can also occur by simply increasing the diameter of the membrane. To demonstrate this effect, we modified a 3.8 cm diameter membrane and compared its performance to 2

cm-diameter membranes (3.6-fold increase in surface area). The water permeability of the larger membrane is $60 \pm 3 \text{ mL}/(\text{cm}^2 \text{ min atm})$, slightly less than that obtained with the 2 cm diameter system ($85 \pm 4 \text{ mL}/(\text{cm}^2 \text{ min atm})$). Within experimental uncertainty, the volume-normalized lysozyme binding capacity measured for the larger membrane ($105 \pm 14 \text{ mg}/\text{cm}^3$) is the same as for the smaller system. Overall, the 3.8 cm-diameter membrane binds 3.3-fold more protein than 2 cm-diameter membranes, and the pressure drops for the two systems differ by only ~20%. Thus increasing the membrane area provides a viable alternative for scaling up separations, as expected.

3.6. HisU binding to poly(MES)-NTA- Ni^{2+} -modified membranes

Affinity purification methods rely on specific interactions between immobilized ligands and affinity tags appended to the protein of interest. Polyhistidine is the most commonly used tag in recombinant protein purification [40], and we employ HisU as a model His-tagged protein to determine the binding capacity of poly(MES)-NTA- Ni^{2+} -modified nylon membranes. Figure 5 shows the breakthrough curve for HisU binding to a poly(MES)-NTA- Ni^{2+} -modified nylon membrane along with similar curves for Con A binding to poly(MES)-NTA- Cu^{2+} and lysozyme binding to poly(MES). Integration of the differences between the feed and the permeate concentrations in the breakthrough curves gives binding capacities of 88 ± 4 , 86 ± 10 and $122 \pm 9 \text{ mg}/\text{cm}^3$ for HisU, Con A and lysozyme, respectively. The permeate flow rate for this analysis was initially 1 mL/min but declined to 0.8 – 0.9 mL/min at the end of the experiment due to the protein loading. (The peristaltic pump could not maintain the flow rate at higher pressure drops.) The similar shapes of the breakthrough curves for all 3 proteins again suggest that the geometrical inhomogeneity of the pore structure in the spongy nylon membrane defines the shape of the curve, rather than binding kinetics or diffusion limitations. The similarity in the curves occurs in spite of the variation in protein size (HisU - 10.7 kDa; Lysozyme – 14.3 kDa; Con A – 104 kDa) and the binding mechanism (HisU and Con A – affinity binding; lysozyme – ion exchange).

After obtaining the breakthrough curve of HisU, we washed the poly(MES)-NTA- Ni^{2+} -HisU membrane with washing buffer II (20 mL) followed by 20 mL of phosphate buffer and eluted the protein with 5–10 mL of elution buffer containing 0.5 M imidazole and 0.5 M NaCl. A Bradford assay of the eluent gives a binding capacity of $96 \pm 7 \text{ mg}/\text{cm}^3$, consistent with the binding capacity value from the breakthrough curve. This binding capacity is 4- to 6-fold higher than those reported for affinity membranes in the literature [19, 41–44] and about twice that of commercial IMAC resins (40–50 mg/mL of resin) [38, 45]. Elution of Con A with phosphate buffer (pH 6) containing 50 mM EDTA, and lysozyme with phosphate buffer (pH 7.4) containing 1 M KSCN gives membrane binding capacities of 88 ± 2 and $136 \pm 4 \text{ mg}/\text{cm}^3$, respectively, again confirming the binding capacity obtained from breakthrough curve analysis and demonstrating efficient elution.

3.7. Con A binding to a monolayer of NTA- Cu^{2+} and other poly(MES)-NTA- Cu^{2+} brushes

A major premise of this work is that polymer brushes greatly increase the binding capacities of membrane adsorbers. To help demonstrate this premise, we deposited NTA complexes in a nylon membrane by linking aminobutyl NTA to a poly(acrylic acid)-terminated polyelectrolyte film (Figure 6, see the supplementary material for the experimental procedure). Covalent attachment of NTA to this surface uses the same reaction as attachment to poly(MES), but the structure of the polyelectrolyte film should only yield approximately a monolayer of NTA.

Figure 7 shows the breakthrough curve obtained for the monolayer of NTA- Cu^{2+} along with the breakthrough curves for Con A binding to poly(MES)-NTA- Cu^{2+} -modified membranes prepared using both the macroinitiator (5-min polymerization) and the trichlorosilane

initiator (1-h polymerization). The Con A binding capacities determined from the curves are 23 ± 8 , 76 ± 6 and 86 ± 10 mg of Con A per cm^3 of membrane for the monolayer, trichlorosilane-based brush and macroinitiator-based brush, respectively. Within experimental error, the capacities determined from the eluate analysis (17 ± 7 , 80 ± 8 and 88 ± 3 mg/cm^3) were the same. Breakthrough curves and the eluent analyses reveal that binding to the brushes is about 4 times higher than to the monolayer, clearly showing the advantage of growing polymer brushes within the pores of the membrane.

The amount of copper present in the NTA monolayer-containing membrane is 2.2 ± 0.3 mg/cm^3 , or 4- to 5-fold less than the amount of Cu^{2+} in the macroinitiator-based poly(MES)-NTA- Cu^{2+} system (10 ± 0.4 mg/cm^3). Thus the mass ratio of protein to Cu^{2+} is around 10:1 for both poly(MES)-NTA- Cu^{2+} and the monolayer of NTA- Cu^{2+} . In contrast, the trichlorosilane-based poly(MES)-NTA- Cu^{2+} contains much more copper (29 ± 4 mg/cm^3) [21] and has about a 3-fold lower ratio (3:1) of protein to Cu^{2+} than the macroinitiator system. As films increase in thickness, access to Cu^{2+} complexes apparently decreases.

Bovine serum albumin (BSA) is commonly used to examine protein binding to Cu^{2+} complexes. BSA binding to the macroinitiator-based system was quite low (~ 30 mg/cm^3), and we were unable to reproduce the BSA binding capacities reported earlier for the trichlorosilane based system, even though lysozyme and HisU binding were reproducible. Since SDS-PAGE of BSA showed a number of bands, we selected Con A rather than BSA for this work.

3.8. Purification of His-tagged MIPS and HisU from cell extracts and protein mixtures

The above studies reveal the high binding capacity of membranes modified with poly(MES)-NTA- Ni^{2+} but do not address selectivity. To demonstrate that membranes can isolate His-tagged protein from complex cell extracts, we purified His-tagged MIPS that was over-expressed in *E. coli*. Figure 8(a) shows the SDS-PAGE analysis of the cell extract (lane 1) and the eluate from a membrane loaded with the cell extract and rinsed with buffers (lane 2). Remarkably, the eluate contains a single detectable band. The total time for purification of 5 mL of cell extract was about 13 min, including 3 min and 20 sec to flow the extract through the membrane at 1.5 mL/min, 4 min (each) to rinse the membrane with washing buffer II and III at 5 mL/min, and 1 min and 20 sec for elution at 1.5 mL/min.

In addition to selectivity, high protein recoveries are vital when purifying tagged proteins. Unfortunately, in most purifications of overexpressed recombinant proteins from cell lysates, ignorance of the initial protein concentration prevents calculation of recovery. Thus, to determine recovery with a poly(MES)-NTA- Ni^{2+} -modified nylon membrane, we prepared a protein mixture containing BSA, ovalbumin, Con A, myoglobin and HisU in equal amounts (0.05 mg/mL of each in 20 mM phosphate buffer). This mixture (10 mL) was passed through the poly(MES)-NTA- Ni^{2+} -modified membrane at 1.5 mL/min, followed by rinsing with washing buffer II and phosphate buffer, and elution of the bound protein with 2 mL of phosphate buffer containing 0.5 M imidazole and 0.5 M NaCl. SDS-PAGE of the loading solution effluent suggests selective and complete removal of the HisU (Figure 8(b) – lane 2), whereas similar analysis of the eluate shows a single band due to HisU (Figure 8(b) – lane 3). The high purity of the eluted protein allowed quantitation using a Bradford assay, and the eluted protein recovery on a single membrane was a remarkable 94 ± 4 %. Overall, less than 10% of the protein was lost in the loading, rinsing, and elution steps.

As a more stringent test of recovery, we obtained a crude *E. coli* cell extract void of His-tagged protein, diluted the extract 1:1 with phosphate buffer, spiked it with 2.5 mg of HisU and passed this solution through a poly(MES)-NTA- Ni^{2+} -modified membrane. Following rinsing with washing buffer II and phosphate buffer, we eluted the bound protein with a

phosphate buffer containing 0.5 M imidazole and 0.5 M NaCl. Gel electrophoresis confirmed the purity of the eluted protein (Figure S-7 – lane 3), and a Bradford assay of the eluate gave a recovery of 91 ± 2 % for this membrane. The high recovery occurs even when the total HisU binding (2.5 mg) approaches the membrane capacity (~ 3 mg).

3.9. Polymer growth and protein binding in other polymeric membranes

Macroinitiator adsorption and MES polymerization occur in aqueous solutions, so growth of poly(MES) brushes should be possible in a wide range of polymeric membranes. To examine the versatility of this method, we attempted to grow poly(MES) in PES, PVDF, non-hydroxylated nylon, and regenerated cellulose membranes and studied lysozyme binding to these brushes. We could not modify the regenerated cellulose because it became very fragile after exposure to water. Figures S-8 (a), (b), and (c) show the ATR-IR spectra of PES, PVDF and non-hydroxylated nylon membranes, respectively, before and after growth of poly(MES). We slightly modified the membrane modification procedure in these cases to include deposition of a layer of PSS prior to the attachment of the macroinitiator to the membrane. PSS sticks well to many surfaces and provides a negatively charged substrate for macroinitiator adsorption. Also, we added the polymerization catalyst directly to the monomer/solvent mixture during the degassing process to avoid the use of any DMF. DMF may damage membranes, particularly PES. On hydroxylated nylon membranes, this altered procedure yields a lysozyme binding capacity of 119 mg/cm^3 , showing that the changes to the process do not affect the polymer brush growth or protein binding. The lysozyme binding capacities obtained from breakthrough curves for PES, PVDF and nonhydroxylated nylon membranes were 65 mg/cm^3 , 64 mg/cm^3 and 115 mg/cm^3 respectively. Thus, the completely aqueous polymerization can modify a wide variety of polymeric membranes, but it may be most effective on nylon. Binding capacity will vary with membrane geometry as well as the effectiveness of poly(MES) growth.

4. Conclusions

Polymerization of MES from immobilized macroinitiators in porous membranes is a rapid method (10 min of initiator attachment and 5 min of polymerization) for synthesizing high-capacity, protein-binding membranes that are highly permeable. Poly(MES)-modified nylon membranes bind $122 \pm 9 \text{ mg/cm}^3$ of lysozyme, and after further derivatization to poly(MES)-NTA- Ni^{2+} the membranes bind $88 \pm 4 \text{ mg/cm}^3$ of HisU. Moreover lysozyme capture in these membranes can occur during a ~ 35 ms residence time. Macroinitiator adhesion to hydroxylated nylon is sufficient to allow surface-initiated polymerization as well as repetition of lysozyme-binding experiments 5 times on the same membrane without a significant decrease in binding capacity. Nevertheless, given the physical nature of macroinitiator adsorption, membrane stability needs further investigation. The HisU binding capacity is twice as high as commercial resin packed columns. Additionally poly(MES)-NTA- Ni^{2+} -modified membranes allow purification of His-tagged MIPS directly from cell extracts in less than 15 min using a simple peristaltic pump. Remarkably, the recovery of HisU from a cell extract is ~ 90 %. Scale up of the protein separation can occur both by stacking several membranes and increasing the membrane diameter. The polymerization method is completely aqueous and thus compatible with nylon (hydroxylated and non-hydroxylated), PES, PVDF and presumably other polymeric membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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 OpenDocument&parentid=525604&moduleid=165902&zone=Labsep (Dynamic binding capacity Approx. 40 mg histidine-tagged protein/ml medium; Max. linear flow rate 600 cm/h)
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<http://www.qiagen.com/products/protein/purification/qiaexpressproteinpurificationsystem/nitaaagrose.aspx?ShowInfo=1> (Binding capacity Approx. 50 mg histidine-tagged protein/mL medium)

Highlights

- Macroinitiator adsorption allows aqueous growth of polymer brushes in 5 min.
- Proteins bind to highly permeable, brush-modified membranes in 35 ms.
- Modified membranes exhibit high protein-binding capacities (100 mg/cm³).
- Brushes containing Ni²⁺ isolate >90% of a His-tagged protein from a cell extract.

Figure 1.

Schematic showing macroinitiator immobilization within membrane pores, polymerization of MES from an initiator-modified membrane, derivatization of poly(MES) with NTA-Ni²⁺ complexes and protein binding to such polymer brushes.

Figure 2. Cu^{2+} binding capacities of poly(MES)-NTA-modified nylon membranes as a function of polymerization time. Poly(MES) was grown from adsorbed macroinitiator.

Figure 3.

Evolution of the lysozyme binding capacity (red squares) and hydraulic permeability (blue diamonds) of nylon membranes with the time allowed for growth of poly(MES) brushes. Hydraulic permeability was obtained at a pressure of 0.68 atm, and binding amounts were determined from breakthrough curves during passage of 15 mL of 0.3 mg/mL lysozyme solution through the membrane.

Figure 4. Breakthrough curves for the passage of 0.3 mg/mL lysozyme through a poly(MES)-modified nylon membrane at flow rates of 1 mL/min (blue diamonds) and 30 mL/min (red squares). Similar curves were obtained for two sets of membranes.

Figure 5.

Breakthrough curves for adsorption of lysozyme (blue diamonds), HisU (green triangles) and Con A (red squares) in nylon membranes modified with poly(MES) (lysozyme), poly(MES)-NTA-Ni²⁺ (HisU) or poly(MES)-NTA-Cu²⁺ (Con A) brushes. The feed solution contained 0.3 mg protein/mL, and the flow rate was between 0.8 and 1.0 mL/min.

Figure 6.

Schematic of protein binding to a monolayer of NTA- Cu^{2+} linked to a polyelectrolyte multilayer adsorbed within the pores of a membrane.

Figure 7.

Breakthrough curves for binding of Con A to a monolayer of NTA-Cu²⁺ (blue diamonds), a trichlorosilane initiator-based poly(MES)-NTA-Cu²⁺-modified membrane (red squares), and a macroinitiator-based poly(MES)-NTA-Cu²⁺-modified membrane (green triangles). The feed solution contained 0.1 mg/mL Con A.

Figure 8. SDS-PAGE analysis (silver staining) of (a) an extract from *E. Coli* containing over expressed His-tagged MIPS (lane 1) and His-tagged MIPS purified from the cell extract using a poly(MES)-NTA- Ni^{2+} -modified membrane (lane 2); (b) a mixture of BSA, ovalbumin, Con A, myoglobin and HisU (lane 1), the effluent from loading the membrane with this solution (lane 2), and the eluate from the membrane (lane 3).