High-Capacity Purification of His-tagged Proteins by Affinity Membranes Containing Functionalized Polymer Brushes

Parul Jain, Lei Sun, Jinhua Dai, Gregory L. Baker,* and Merlin L. Bruening*

Department of Chemistry, Michigan State University, East Lansing, Michigan 48824

Received May 10, 2007; Revised Manuscript Received July 26, 2007

Porous membrane absorbers are attractive for increasing the rate of protein purification, but their binding capacity is low relative to porous beads. Modification of membranes with functionalized polymer brushes, however, can greatly enhance capacity. This work demonstrates that membrane modification with poly(2-hydroxyethyl methacrylate) (PHEMA) brushes derivatized with nitrilotriacetate−Ni^{2+} (NTA−Ni^{2+}) complexes allows purification of polyhistidine-tagged ubiquitin (HisU) in less than 30 min with a binding capacity of 120 mg of HisU/cm^3 of porous alumina membrane. Adsorption isotherms show that saturation of the brushes occurs at HisU concentrations as low as 0.04 mg/mL and that these brushes can bind up to 23 monolayers of HisU. Gel electrophoresis reveals that the purity of eluted HisU is more than 99%, even when the initial feed solution contains 10% bovine serum or a 20-fold excess of BSA. Thus, reusable porous membranes modified by PHEMA−NTA−Ni^{2+} brushes are attractive candidates for rapid purification of polyhistidine-tagged proteins.

Introduction

Advances in genetic engineering have generated an ongoing need for more efficient purification of recombinant proteins.[1,2] One of the most powerful methods for isolating such proteins, affinity chromatography, is based on specific interactions between immobilized ligands and an affinity tag (e.g., polyhistidine,[3] glutathione-S-transferrase (GST),[4] streptavidin,[5] or maltose binding protein (MBP)[6]) that is appended to the protein of interest. Polyhistidine, the most frequently employed tag, allows purification by immobilized metal-affinity chromatography (IMAC),[7−10] where selectivity is usually based on the interaction of the polyhistidine with an immobilized Ni^{2+} complex. Proteins containing a polyhistidine tag (his tag) are selectively bound to the chromatographic matrix, while other cellular proteins are washed away.[11]

The advantages of IMAC, which include ligand stability, high protein loading, mild elution conditions, simple regeneration and low cost,[12] are very important when developing purification procedures. Nevertheless, this technique has some serious limitations, including long separation times, difficulties in packing large columns, relatively high pressure drops, and slow intrabead diffusion of solutes.[13−15] These limitations will be particularly important for large-scale separations. (The use of commercially available Gravity-flow and Spin-trap columns allows some reduction in purification times for small-scale separations.[16])

In an effort to address some of the challenges that are general to affinity chromatography, membrane chromatography was first introduced in 1988.[17] In this technique, the flow of solution through membrane pores containing immobilized ligands enhances the rate of mass transport to the ligands.[18−22] Increasing the scale of such separations can be accomplished relatively easily by increasing membrane surface area.

Despite their potential, however, the major disadvantage of membrane absorbers is that their low internal surface area (when compared to porous beads) yields a relatively low binding capacity. To overcome this problem, we and others are developing membranes modified with polymer brushes that have multiple protein-binding sites (Figure 1).[23−29] The controlled growth of polymers in membrane pores using atom transfer radical polymerization (ATRP) is particularly attractive because the thickness of the resulting polymer brushes can be readily controlled to optimize capacity without completely filling pores. In a previous study, we demonstrated growth of poly(2-hydroxyethyl methacrylate) (PHEMA) from initiators bound to a porous alumina membrane and subsequent functionalization of the PHEMA with nitrilotriacetate−Cu^{2+} (NTA−Cu^{2+}) complexes.[25] The resulting membranes bound large amounts of BSA and myoglobin, presumably via formation of complexes between the histidine groups of the proteins and the immobilized NTA−Cu^{2+}. Overall, the combination of a porous alumina support and functionalized polymer brushes yielded a remarkable binding capacity of more than 100 mg of BSA/cm^3 of membrane.[25] However, the high BSA-binding capacity suggests that membranes containing PHEMA−NTA−Cu^{2+} are prone to binding of many proteins and that NTA−Cu^{2+} is not sufficiently selective to effectively purify his-tagged proteins.

This research describes the use of PHEMA−NTA−Ni^{2+}, rather than PHEMA−NTA−Cu^{2+}, brushes to modify membranes and selectively purify his-tagged proteins. It is well-known that NTA−Ni^{2+} is highly selective for binding of his-tagged proteins because the relatively weak interaction of NTA−Ni^{2+} with histidine requires a polyhistidine for efficient binding (Figure 1).[3,30] The PHEMA−NTA−Ni^{2+}-modified membranes described here allow rapid purification of his-tagged ubiquitin (HisU) from a 20-fold excess of BSA as well as from a mixture containing 10% bovine serum and 0.3 mg/mL HisU. Gel electrophoresis reveals that the purity of HisU recovered from such solutions is >99%, and the binding capacity of these membranes is as high as 120 mg of HisU/cm^3 of membrane. Separations can be completely performed in 30 min or less, and membranes are fully reusable.

Experimental Section

Materials. Anodisc porous alumina membranes with 0.2 μm-diameter surface pores were obtained from Fisher Scientific. Dimeth-

* Authors to whom correspondence should be addressed. E-mail: bruening@chemistry.msu.edu; bakerg@msu.edu. Phone: (517) 355−9715 ext. 237. Fax: (517) 353−1793.

10.1021/bm700515m CCC: $37.00
Published on Web 09/19/2007

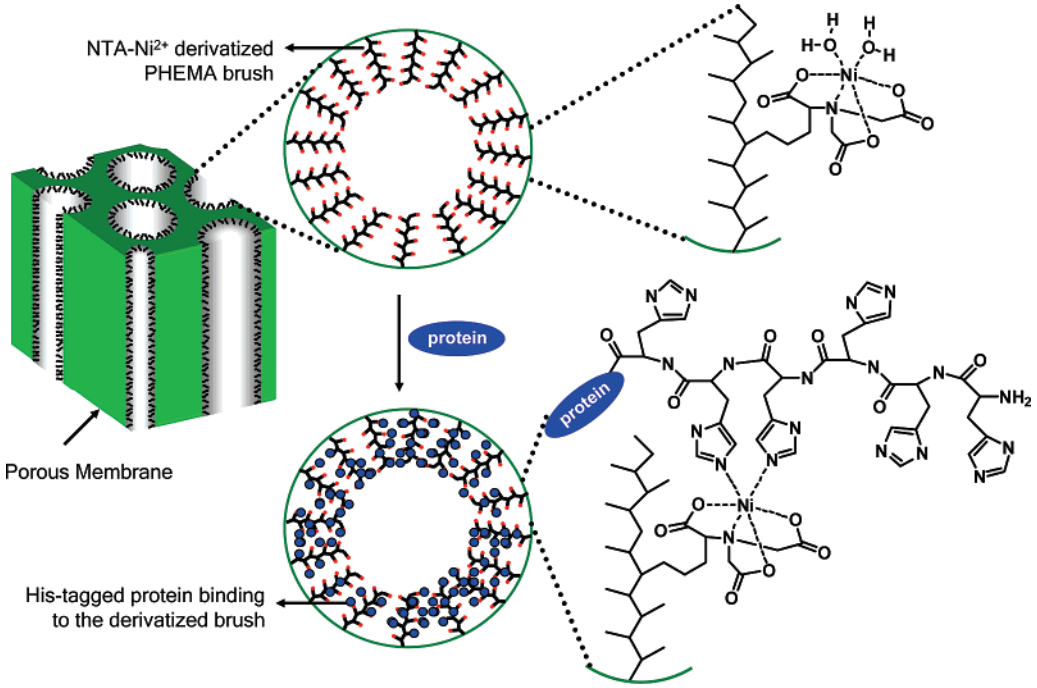


Figure 1. Binding of his-tagged protein to a NTA−Ni^{2+} derivatized PHEMA brush inside a membrane pore.

ylformamide (DMF, anhydrous, 99.8%), 11-mercaptoundecanol (97%), 2-bromoisobutyryl bromide (98%), CuCl (99.999%), $CuBr_2$ (99%), 2,2′-bipyridyl (bpy, 99%), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 4-dimethylaminopyridine (DMAP), ethylenediamine tetraacetic acid (EDTA), imidazole (99%), TWEEN-20 surfactant, bovine serum albumin (BSA), lysozyme, ubiquitin, N-terminal $histidine_6$ tagged ubiquitin (HisU), and myoglobin were used as received from Sigma Aldrich. Fetal bovine serum was obtained from HyClone. $NiSO_4 \cdot 5H_2O$ (Columbus Chemical), NaH_2PO_4 (CCI), Na_2HPO_4 (Aldrich), N_α,N_α-bis(carboxymethyl)-L-lysine hydrate (Fluka, aminobutyl NTA), succinic anhydride (SA, Matheson Coleman & Bell), and Coomassie protein assay reagent (Pierce) were also used without purification. 2-Hydroxyethyl methacrylate (HEMA, Aldrich, 97%, inhibited with 300 ppm hydroquinone monomethyl ether) was purified by passing it through a column of activated basic alumina (Aldrich), and trichlorosilane initiator (11-(2-bromo-2-methyl)propionyloxy)undecyltrichlorosilane) was synthesized according to a literature procedure.[31] Buffers were prepared using analytical grade chemicals and deionized (Milli-Q, 18.2 MΩ cm) water.

Polymerization of 2-Hydroxyethyl Methacrylate (HEMA) in Porous Alumina Membranes and on Au Substrates. The procedure for polymerizing HEMA inside alumina membranes was reported previously.[25] Briefly, the alumina membrane was sandwiched inside a membrane cell (Millipore, Swinnex 25), and a solution of the trichlorosilane initiator, (11-(2-bromo-2-methyl)propionyloxy)undecyltrichlorosilane, was passed through the membrane followed by subsequent rinsing. Polymerization of HEMA from the immobilized initiator occurred by circulating a degassed solution containing 15 mL of purified HEMA, 15 mL of methanol, 82.5 mg (0.825 mmol) of CuCl, 54 mg (0.24 mmol) of $CuBr_2$, and 320 mg (2.04 mmol) of 2,2′-bipyridyl through the initiator-modified membrane for 1 h. (The use of a mixed halide system sometimes provides better control over polymerization.[32,33]) After polymerization, the membrane was cleaned with flowing ethanol (20 mL), deionized water (Milli-Q, 18.2 MΩ cm, 20 mL), and acetone (20 mL).

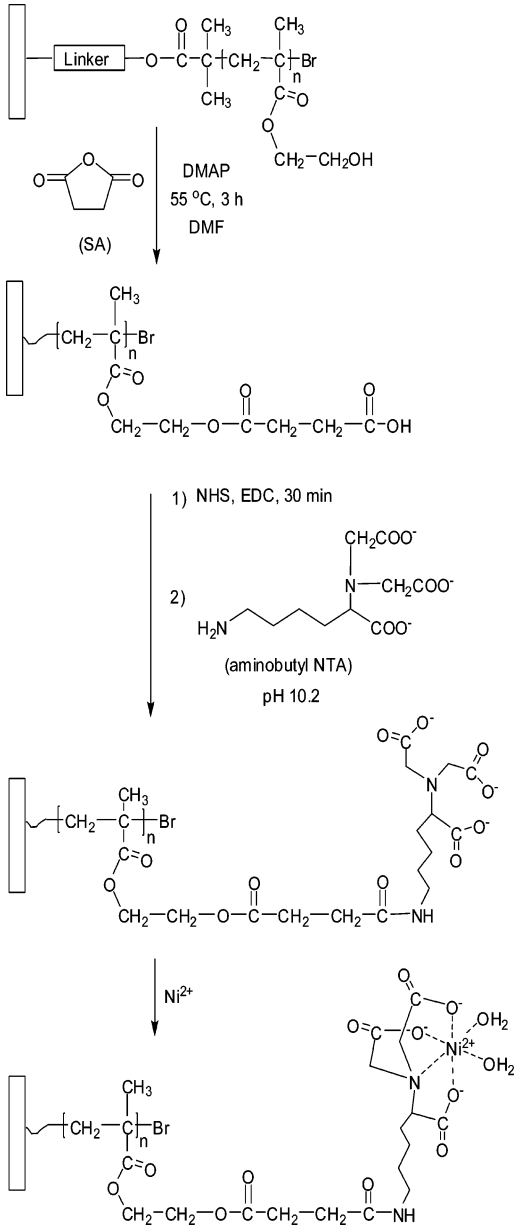
To prepare films for reflectance FTIR and ellipsometric characterization, Au-coated silicon wafers were coated with a mercaptoundecanol monolayer that was subsequently allowed to react with 2-bromoisobutyryl bromide as described previously.[33,34] Polymerization of HEMA from these substrates occurred as described above, except that the substrate was simply immersed in a polymerization solution that was kept in a glove bag, and water was used instead of methanol to give thicker films.[34]

PHEMA Derivatization and Protein Immobilization. The derivatization procedure was also described previously and is shown in Scheme 1.[25] However, in the present case, the aminobutyl NTA derivatized membrane was exposed to a 0.1 M $NiSO_4$, rather than 0.1 M $CuSO_4$, solution and rinsed with buffer. After loading of membranes with Ni^{2+}, a solution containing pure protein or a mixture of proteins (in 20 mM phosphate buffer, pH 7.2) was then pumped through the membrane using a peristaltic pump, and the permeate was collected for analysis at specific time intervals. Subsequently, the membrane was rinsed with 20 mL of pH 7.2 washing buffer (20 mM phosphate buffer containing 0.1% Tween-20 surfactant and 0.15 M NaCl) and 20 mL of phosphate buffer, and protein was then eluted using 5−10 mL of a solution containing 20 mM sodium phosphate, 0.5 M NaCl, and 0.5 M imidazole at pH 7.4. Ni^{2+} was later eluted using a 50 mM EDTA solution (pH 7.2), and the PHEMA-NTA film was recharged with Ni^{2+} prior to reuse. To prepare derivatized films for reflectance FTIR characterization, a PHEMA film on a gold substrate was treated in a similar procedure by immersing the substrate in appropriate solutions and rinsing with solutions (20 mL each) from a pipet.

Film Characterization Methods. FTIR spectra of films on gold-coated wafers were obtained with a Nicolet Magna-IR 560 spectrometer containing a PIKE grazing angle (80°) accessory, and film thicknesses were measured using a rotating analyzer ellipsometer (model M-44; J.A. Woollam) at an incident angle of 75°, assuming a film refractive index of 1.5. Ellipsometric measurements were performed on at least three spots on a film. A UV/ozone-cleaned gold-coated wafer was used as a background for reflectance FTIR spectra. Film growth inside alumina membranes was verified using transmission FTIR spectroscopy (Mattson Galaxy Series 3000) with an air background.

Determination of the Amount of Coordinated Ni^{2+} in the Membrane. A calibration curve was determined by measuring the absorbance of $NiSO_4$ standard solutions in 50 mM EDTA (pH 7.2) using a Varian Spectra AA-200 atomic absorption spectrophotometer, and a sample solution was obtained by eluting Ni^{2+} from a PHEMA−NTA−Ni^{2+}-coated membrane with 5.0 mL of 50 mM EDTA (pH 7.2).

Scheme 1. Derivatization of PHEMA with NTA−Ni^{2+} Prior to Protein Adsorption



The amount of Ni^{2+} in the stripping solution was calculated from its absorbance using the calibration curve.

Protein Quantification. To determine the concentration of protein bound to PHEMA brushes in a membrane, 50 μL of eluent was added to 2.95 mL of a solution of Coomassie reagent, and the mixture was shaken a few times and allowed to react for 5 min at room temperature. The UV/vis absorbance spectra of these solutions were then obtained with a Perkin-Elmer UV/vis (model Lambda 40) spectrophotometer. Calibration curves for the absorbance of BSA, HisU, and myoglobin solutions at 595 nm were prepared using a series of protein solutions (concentration range of 100 μg to 1 mg of protein per mL) that were mixed with Coomassie reagent. All spectra were measured against a Coomassie reagent background.

To quantify the amount of protein bound to PHEMA brushes on gold-coated Si, the method reported previously by Dai and co-workers was employed.[23] Briefly, a calibration curve was obtained by plotting the ellipsometric thickness of spin-coated BSA, myoglobin, lysozyme, or ubiquitin films against the reflectance FTIR absorbance of their amide I band.

Determination of Protein Purity by SDS-PAGE. The protein solutions were analyzed by SDS-PAGE with a 16% cross-linked separating gel and a 4% cross-linked stacking gel (acrylamide). Protein bands were visualized using a standard silver staining procedure.[15]

Results and Discussion

Characterization of PHEMA-Derivatized Membranes. To form brush-modified membranes, PHEMA was first grown from ATRP initiators that were immobilized within porous alumina via silanization.[25] The brushes were derivatized as shown in Scheme 1, with reactant solutions being flowed through the membrane using a peristaltic pump. Transmission IR spectra (Figure 1, Supporting Information) of membranes after HEMA polymerization and each derivatization step provide evidence for formation of PHEMA, subsequent reaction with succinic anhydride, and finally successful linking of aminobutyl NTA to these polymer brushes. These transmission IR spectra are consistent with previous spectra of PHEMA films grown on Au-coated Si.[25]

To determine the amount of Ni^{2+} present in PHEMA−NTA−Ni^{2+} brushes inside alumina membranes, we passed 5 mL of 50 mM EDTA through a modified membrane and subsequently analyzed the EDTA solution for Ni^{2+} using atomic absorption spectroscopy. This procedure revealed that 14 ± 1 μmol Ni^{2+} was present in a 2-cm-diameter, 60-μm-thick modified membrane. Assuming that every NTA moiety bound a Ni^{2+} ion and that each hydroxyl group of PHEMA was derivatized with one NTA moiety, these results suggest that prior to derivatization the PHEMA consisted of 12-nm-thick annuli inside the 230-nm-diameter pores in the alumina. (This calculation also assumes a PHEMA density of 1 g/cm^3 and a membrane porosity of 50%.)

HisU Binding to PHEMA−NTA−Ni^{2+} Brushes on Gold Substrates. We first examined absorption of his-tagged proteins in films formed on Au-coated Si to allow characterization of binding using ellipsometry and reflectance FTIR spectroscopy. These films were grown from monolayers of initiator as reported previously.[35] In initial studies, absorption of HisU occurred during a 2-h immersion of the PHEMA−NTA−Ni^{2+}-modified gold substrate in a stirred solution containing 0.01 mg/mL HisU in pH 7.2 phosphate buffer. The film was then rinsed with 20 mL of washing buffer (20 mM phosphate buffer containing 0.1% Tween-20 surfactant, and 0.15 M NaCl), immersed in phosphate buffer (pH 7.2) for 15 min, and finally rinsed with ethanol. To examine whether protein binds to PHEMA−NTA−Ni^{2+}, the reflectance FTIR spectrum of the film prior to protein exposure was subtracted from the corresponding spectrum measured after protein exposure and rinsing. The subtracted spectra of films immersed in 0.01 mg/mL HisU show strong amide I (1680 cm^{-1}) and amide II (1545 cm^{-1}) bands (Figure 2, spectrum a), indicative of extensive protein binding. (The small negative peak at 1740 cm^{-1} likely results from deprotonation of some −COOH groups.) In contrast to HisU, the subtracted spectrum of a PHEMA−NTA−Ni^{2+} coating exposed to 0.1 mg/mL BSA for 6 h (Figure 2, spectrum b) shows no amide absorbances, even though the concentration of BSA in the exposure solution was 10-fold more than the concentration used for HisU. To further demonstrate that histidine tags are crucial for binding to PHEMA−NTA−Ni^{2+} brushes, we immersed a film in a 0.1 mg/mL ubiquitin solution in phosphate buffer (pH 7.2) and stirred the solution for 6 h. No ubiquitin binding was detected (Figure 2, spectrum c). Thus, these films exhibit no detectable nonspecific adsorption and should maintain the high selectivities that are available through adsorption of his-tagged proteins to NTA−Ni^{2+} complexes.

Previously, we correlated the subtracted-spectrum amide absorbance of BSA with the amount of BSA bound to

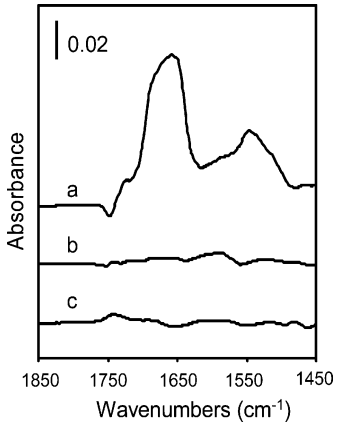


Figure 2. Subtracted reflectance FTIR spectra of protein immobilized on PHEMA−NTA−Ni^{2+} brushes after exposure of the films to (a) 0.01 mg/mL HisU, (b) 0.1 mg/mL BSA, or (c) 0.1 mg/mL ubiquitin. The spectra were obtained by subtracting the spectrum of PHEMA−NTA−Ni^{2+} from that of PHEMA−NTA−Ni^{2+}−protein (both films were rinsed with buffer and ethanol prior to the measurement).

PHEMA−NTA−Cu^{2+} films using calibration curves of ellipsometric thickness versus amide absorbance for films of pure, spin-coated BSA.[23] However, obtaining a calibration curve by spin-coating of HisU is not practical due to the high cost of this protein. To overcome this challenge, we showed that calibration curves of thickness versus amide I absorbance are essentially independent of the protein employed to obtain the curve (Figure 2, Supporting Information). The slopes of calibration curves for BSA, myoglobin, lysozyme, and ubiquitin absorption were all 0.0017 absorbance units/nm protein. The uniformity of slopes is reasonable, as the extinction coefficient for the amide absorbance should be similar for all proteins. Given the value of 0.0017 absorbance units/nm, spectrum a in Figure 2 suggests that the PHEMA−NTA−Ni^{2+} film absorbed 31 nm of HisU. This corresponds to binding of 3.1 μg of HisU/cm^2 or about 10 monolayers of HisU (assuming a HisU density of 1 g/cm^3 and a monolayer thickness of 2.9 nm).[36]

Figure 3 shows how the amount of HisU bound to PHEMA−NTA−Ni^{2+} films on Au varies as a function of (a) concentration and (b) time. (Data were obtained using a combination of reflectance FTIR spectroscopy and calibration curves of thickness versus amide absorbance as described above.) Figure 3a reveals that significant HisU binding occurs at concentrations as low as 0.001 mg/mL and that saturation of the film is approached when HisU concentrations reach 0.04 mg/mL. The data in Figure 3b demonstrate ~90% film saturation in 20 min when using a 0.05 mg/mL HisU solution. The maximum binding capacity of these 51-nm-thick (before derivatization) films was 40 nm of HisU or about 13 monolayers. Capacity increases with film thickness (Figure 3, Supporting Information) but eventually plateaus when PHEMA thickness reaches 115 nm. The highest binding capacity obtained was 70 nm of HisU or the equivalent of about 23 monolayers.

HisU Binding to PHEMA−NTA−Ni^{2+} Brushes in Membranes. To test the HisU binding capacity of PHEMA−NTA−Ni^{2+} brushes in membranes, we pumped a solution of 0.3 mg/mL HisU in pH 7.2 phosphate buffer through the membrane, collected this permeate over specific time intervals, and analyzed these samples using a Bradford assay. Figure 4 shows the breakthrough curve for HisU binding to PHEMA−NTA−Ni^{2+} brushes along with similar curves for BSA and myoglobin binding to PHEMA−NTA−Cu^{2+}-modified membranes. (PHEMA−NTA−Ni^{2+} brushes do not bind a significant amount of BSA and myoglobin as shown below.) Integration of the

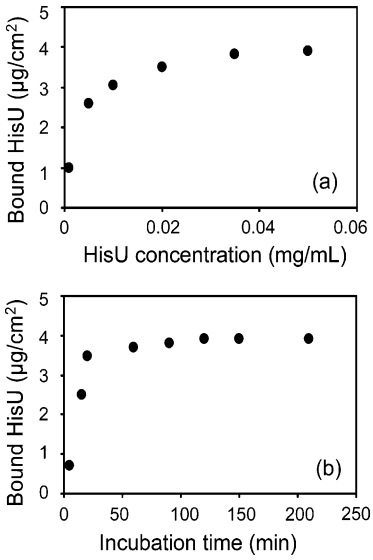


Figure 3. Amount of HisU bound to PHEMA−NTA−Ni^{2+} films as a function of (a) concentration and (b) time. To obtain the data in panel a, a 51 nm-thick (prior to derivatization) PHEMA film was used, and the binding capacity was determined after 2 h. The same PHEMA−NTA film was used throughout the experiment, with protein elution and reloading of Ni^{2+} after measurements at each concentration. For panel b, 0.05 mg/mL HisU and a single 51 nm PHEMA film were used. To test the reproducibility of the experiments, binding on a 51 nm film with a protein concentration of 0.01 mg/mL was repeated three times and found to be 3.01 ± 0.05 μg/cm^2.

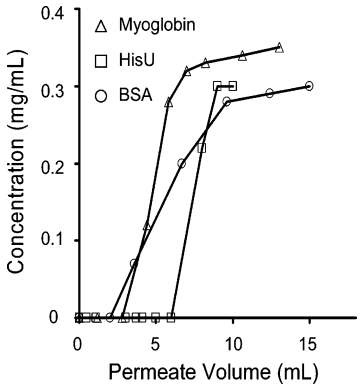


Figure 4. Breakthrough curves for absorption of 0.3 mg/mL HisU, 0.3 mg/mL BSA, and 0.35 mg/mL myoglobin in membranes modified with PHEMA−NTA−Ni^{2+} (HisU) or PHEMA−NTA−Cu^{2+} (BSA and myoglobin). The permeate flow rate was initially 2.4 and 0.9 mL/min at the end of the experiment.

differences between the feed concentrations and the permeate concentrations shown in the breakthrough curves gives binding capacities of 110 and 83 mg/cm^3, respectively, for binding of BSA and myoglobin to PHEMA−NTA−Cu^{2+}-modified membranes and 120 mg/cm^3 for HisU binding to a PHEMA−NTA−Ni^{2+}-modified membrane. However, the breakthrough curves of HisU and myoglobin are slightly sharper than that for BSA. (Constant-pressure measurements showed that flow rates are similar for all three proteins for a given permeate volume.) The very sharp breakthrough curve for HisU may stem from its high affinity for the NTA−Ni^{2+} or its low molecular mass (10.7 kDa) relative to myoglobin (17 kDa) and BSA (67.5 kDa). A low molecular mass could allow rapid diffusion of smaller proteins into the polymer brushes, but future studies with several different his-tagged proteins are needed to determine if this is the case.

The linear flow velocity through the membrane is on average about 21 cm/h. This is about 1 order of magnitude less than the flow rate through typical gels,[37−40] but the capacity of the

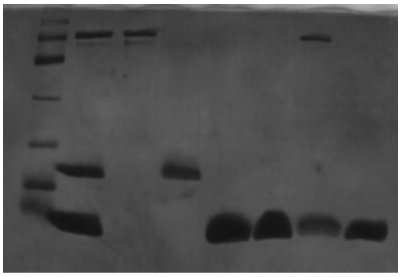


Figure 5. SDS-PAGE analysis (silver staining) of protein solutions and eluents from PHEMA−NTA−Ni^{2+}−alumina membranes. Samples are as follows: lane 1, standard broad range ladder; lane 2, mixture of BSA, myoglobin and HisU (0.05 mg/mL each); lane 3, BSA; lane 4, myoglobin; lane5, HisU; lane 6, eluent from a membrane loaded with a mixture of BSA, myoglobin and HisU (0.05 mg/mL each); lane 7, mixture of BSA and HisU; lane 8, eluent from a membrane loaded with 10 mL of a solution containing 1 mg/mL BSA and 0.05 mg/mL HisU.

membranes per cm^3 is also 1 order of magnitude greater than that of gels.[37−40] Still, linear velocities need to increase to make the membranes a more competitive technology, and this can be accomplished by increasing pore size (at a constant porosity, flow rate is proportional to the square of pore radius for assemblies of parallel pores).

After measuring the breakthrough curve of HisU, the PHEMA−NTA−Ni^{2+}−HisU membrane was washed with 20 mL of washing buffer followed by 20 mL of phosphate buffer, and HisU was eluted with 5−10 mL of elution buffer containing 0.5 M imidazole. Analysis of the eluent using a Bradford assay and comparison of this analysis with the capacity determined from the breakthrough curve showed that 99 ± 1% of the bound HisU was recovered.

To prove that PHEMA−NTA−Ni^{2+}-modified membranes selectively bind HisU, we also investigated the binding of BSA and myoglobin to PHEMA−NTA−Ni^{2+} films in alumina. A PHEMA−NTA−Ni^{2+}-derivatized membrane was loaded with 10 mL of 0.2 mg/mL BSA or 10 mL of 0.2 mg/mL myoglobin in buffer, rinsed with washing buffer, and treated with eluent. BSA was not detected in the imidazole eluent (<0.001 mg/cm^3), whereas a small amount (0.05 mg/cm^3) of myoglobin binding was observed. However, the amount of myoglobin bound was only 3% of the amount of HisU bound when using 0.3 mg/mL HisU. (A total of 11 of the 153 residues of myoglobin are histidine, which could enhance the undesired adsorption of this protein to NTA−Ni^{2+}).

Separation of Protein Mixtures using PHEMA−NTA−Ni^{2+} Brushes in Membranes. Three sets of experiments were carried out to test the ability of alumina−PHEMA−NTA−Ni^{2+} membranes to purify his-tagged proteins. In the first experiment, 6 mL of phosphate buffer containing BSA, myoglobin, and HisU (0.05 mg/mL of each protein) was passed through the PHEMA−NTA−Ni^{2+}-derivatized membrane, and the membrane was then rinsed with 20 mL of washing buffer followed by 20 mL of phosphate buffer. Finally, we used 5−10 mL of elution buffer to recover the bound protein and analyzed this eluent by electrophoresis. The gel electropherogram of the eluent (Figure 5, lane 6) shows no bands due to BSA or myoglobin and an intense band for HisU, demonstrating that one pass through the membrane is sufficient to give >99% pure HisU (lanes 1−5 are given to show positions of proteins). The purity was calculated assuming that the amount of BSA in the 15 μL protein solution that was loaded on the gel is <20 ng. The concentration of HisU was calculated based on a Bradford assay of the protein sample, which was possible because of the high purity of the eluent.

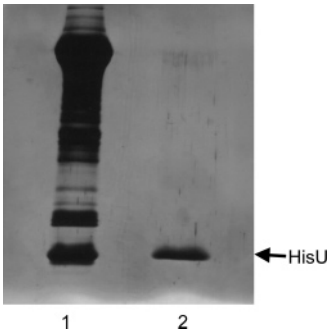


Figure 6. SDS-PAGE analysis (silver staining) of 10% bovine serum spiked with 0.3 mg/mL HisU (lane 1) and the imidazole eluent from an alumina-PHEMA−NTA−Ni^{2+} membrane loaded with this solution (lane 2).

After this initial experiment, we washed the membrane with 10 mL of 50 mM EDTA, rinsed it with water, recharged it with 0.1 M Ni^{2+}, and rinsed with 20 mL of water and 20 mL of phosphate buffer. In a second test of selectivity, 10 mL of phosphate buffer containing 1 mg/mL BSA and 0.05 mg/mL HisU was passed through the membrane, and the membrane was rinsed, treated with elution buffer, and analyzed as described above. Even though this sample contained a 20-fold excess of BSA, the eluent showed only a band for HisU (Figure 5, lane 8).

In a final experiment, 10% Bovine serum in 10 mL of phosphate buffer containing 45 mM imidazole (pH 7.2) was spiked with 0.3 mg/mL HisU. (Imidazole was added to help prevent adsorption of non his-tagged proteins.) This solution was passed through the membrane, which was then rinsed, treated with elution buffer, and analyzed using the procedure described above.

Figure 6 shows the SDS-PAGE analysis of the spiked bovine serum (left) and the eluent from the membrane (right). Although the diluted serum contains about 4 mg/mL of protein and only 0.3 mg/mL of HisU, the electropherogram of the eluent shows a strong band for HisU and only a very faint band of BSA. The HisU in this case is more than 99% pure, assuming that the faint band of BSA represents <40 ng of BSA. (Approximately 4.5 μg of HisU was loaded on the gel, as determined using a Bradford assay of the eluent, and separate gels showed that 40 ng of BSA gave a readily visible band.) Figure 6 clearly shows the selectivity of the membrane for HisU over other proteins present in serum. All of these selectivity experiments were performed twice, and the membranes can be used multiple times.

Conclusions

This work demonstrates that growth of PHEMA−NTA−Ni^{2+} brushes inside porous alumina supports yields high-capacity membranes that selectively bind his-tagged proteins. The membranes show a binding capacity of 120 mg of HisU/cm^3 of membrane along with minimal nonspecific adsorption. Moreover, 99% of the bound HisU could be recovered, and membranes can be reused.

Studies of PHEMA−NTA−Ni^{2+} brushes on flat, gold-coated silicon substrates show that significant HisU binding occurs at concentrations as low as 0.001 mg/mL, and saturation of the film is approached in 20 min or less at HisU concentrations of 0.05 mg/mL. Thus, even at his-tagged protein concentrations

of 0.05 mg/mL, it should be possible to make full use of binding sites in a few minutes. The time required to approach saturation should be even less at higher concentrations and in membranes, where convection through the membrane will help to overcome diffusion limitations.

Gel electrophoresis results indicate that the membrane is selective toward HisU even in the presence of a 20-fold excess of BSA or in 10% bovine serum. The recovered HisU in such cases is 99% pure. These membranes should also be effective for whole-cell extracts provided the solution does not clog the membrane. Increases in membrane pore sizes should help to avoid clogging and increase linear velocities, but large pores may also require some optimization of brush thickness and density to achieve rapid binding and high capacities.

Importantly, the time required for membrane-based purification included only 10 min for loading, 10 min for washing, and 5 min for elution. Thus purification of histidine tagged proteins can be achieved in less than 30 min, and further time reductions could likely be achieved by using more permeable membrane supports and higher pressures. Hence, these membranes are attractive for rapid, selective purification of histidine tagged proteins.

Acknowledgment. We are grateful to the National Science Foundation (CHE-0616795) for funding this work. We also thank Professor Jim Geiger for his help and input.

Supporting Information Available. Transmission IR spectra of PHEMA brushes in porous alumina before and after derivatization steps; reflectance FTIR spectra of spin-coated BSA, myoglobin, lysozyme, and ubiquitin films on gold; and his-tagged ubiquitin binding capacity as a function of brush thickness. This material is free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Arnau, J.; Lauritzen, C.; Peterson, G. E.; Pedersen, J. *Protein Expression Purif.* **2006**, *48*, 1−13.
(2) Zhen, G.; Falconnet, D.; Kuennemann, E.; Voros, J.; Spencer, N. D.; Textor, M.; Zurcher, S. *Adv. Funct. Mater.* **2006**, *16*, 243−251.
(3) Stiborova, H.; Kostal, J.; Mulchandani, A.; Chen, W. *Biotechnol. Bioeng.* **2003**, *82*, 605−611.
(4) Draveling, C.; Ren, L.; Haney, P.; Zeisse, D.; Qoronfleh, M. W. *Protein Expression Purif.* **2001**, *22*, 359−366.
(5) Skerra, A.; Schmidt, T. G. M. *Biomol. Eng.* **1999**, *16*, 79−86.
(6) Cattoli, F.; Boi, C.; Sorci, M.; Sarti, G. C. *J. Membr. Sci.* **2006**, *273*, 2−11.
(7) Gaberc-Porekar, V.; Menart, V. *J. Biochem. Biophys. Methods* **2001**, *49*, 335−360.
(8) Everson, R. J.; Parker, H. E. *Bioinorg. Chem.* **1974**, *4*, 15−20.
(9) Porath, J.; Olin, B. *Biochemistry* **1983**, *22*, 1621−1630.
(10) Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. *Nature* **1975**, *258*, 598−599.
(11) Suen, S.-Y.; Liu, Y.-C.; Chang, C.-S. *J. Chromatogr. B* **2003**, *797*, 305−319.
(12) Özkara, S.; Yavuz, H.; Denizli, A. *J. Appl. Polym. Sci.* **2003**, *89*, 1567−1572.
(13) Zou, H.; Luo, Q.; Zhou, D. *J. Biochem. Biophys. Methods.* **2001**, *49*, 199−240.
(14) Che, S. et al. *Nature* **2004**, *429*, 281−284.
(15) Clairbois, A.-S.; Letourneur, D.; Muller, D.; Jozefonvicz, J. *J. Chromatogr. B* **1998**, *706*, 55−62.
(16) GE Healthcare, Product information on affinity columns. http://www4.amershambiosciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&ParentId=576 (accessed January 5, 2007). The maximum capacity listed was 40 mg/mL.
(17) Brandt, S.; Goffe, R. A.; Kessler, S. B.; O'Connor, J. L.; Zale, S. E. *Biotechnology* **1988**, *6*, 779−782.
(18) Sorci, M.; Boi, C.; Facchini, R.; Sarti, G. C. *Desalination* **2006**, *199*, 550−552.
(19) Endres, H. N.; Johnson, J. A. C.; Ross, C. A.; Welp, J. K.; Etzel, M. R. *Biotechnol. Appl. Biochem.* **2003**, *37*, 259−266.
(20) Boi, C.; Facchini, R.; Sorci, M.; Sarti, G. C. *Desalination* **2006**, *199*, 544−546.
(21) Yang, H.; Viera, C.; Fischer, J.; Etzel, M. R. *Ind. Eng. Chem. Res.* **2002**, *41*, 1597−1602.
(22) Boi, C.; Francesca, C.; Facchini, R.; Sorci, M.; Sarti, G. C. *J. Membr. Sci.* **2006**, *273*, 12−19.
(23) Dai, J.; Bao, Z.; Sun, L.; Hong, S. U.; Baker, G. L.; Bruening, M. L. *Langmuir* **2006**, *22*, 4274−4281.
(24) Kawai, T.; Saito, K.; Lee, W. *J. Chromatogr. B* **2003**, *790*, 131−142.
(25) Sun, L.; Dai, J.; Baker, G. L.; Bruening, M. L. *Chem. Mater.* **2006**, *18*, 4033−4039.
(26) Ulbricht, M.; Yang, H. *Chem. Mater.* **2005**, *17*, 2622−2631.
(27) Yusof, A. H. M.; Ulbricht, M. *Desalination* **2006**, *200*, 462−463.
(28) Hicke, H.-G.; Becker, M.; Paulke, B.-R.; Ulbricht, M. *J. Membr. Sci.* **2006**, *282*, 413−422.
(29) Singh, N.; Husson, S. M.; Zdyrko, B.; Luzinov, I. *J. Membr. Sci.* **2005**, *262*, 81−90.
(30) Johnson, R. D.; Arnold, F. H. *Biotechnol. Bioeng.* **1995**, *48*, 437−443.
(31) Matyjaszewski, K.; Miller, P. J.; Shukla, N.; Immaraporn, B.; Gelman, A.; Lukola, B. B.; Siclovan, T. M.; Kickelbick, G.; Vallant, T.; Hoffmann, H.; Pakula, T. *Macromolecules* **1999**, *32*, 8716−8724.
(32) Matyjaszewski, K.; Shipp, D. A.; Wang, J. -L.; Grimaud, T.; Patten, T. E. *Macromolecules* **1998**, *31*, 6836−6840.
(33) Huang, W.; Kim, J. -B.; Bruening, M. L.; Baker, G. L. *Macromolecules* **2002**, *35*, 1175−1179.
(34) Kim, J.-B.; Huang, W.; Bruening, M. L.; Baker, G. L. *Macromolecules* **2002**, *35*, 5410−5416.
(35) Kim, J.-B.; Bruening, M. L.; Baker, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 7616−7617.
(36) Cook, W. J.; Suddath, F. L.; Bugg, C. E.; Goldstein, G. *J. Mol. Biol.* **1979**, *130*, 353−355.
(37) Talon products for polyhistidine-tagged protein purification. http://wolfson.huji.ac.il/purification/PDF/Tag_Protein_Purification/Ni-NTA/Clontech_Talon_products.pdf (accessed July 5, 2007). Maximum linear flow rate reported was 30 cm/h.
(38) Adar Biotech, Product information on nickel beads for IMAC. http://www.adarbiotech.com/products/instructions/Nickel%20beads%20Instructions.pdf (accessed July 5, 2007). Maximum linear flow rate reported was 240 cm/h.
(39) Sigma-Aldrich His-Select quick reference guide. http://www.sigmaaldrich.com/sigma/general%20information/web%20his%20selection%20guide.pdf (accessed July 5, 2007). Maximum linear flow rate reported was 150 cm/h.
(40) Amersham Biosciences instruction guide for Chelating Sepharose Fast Flow. http://www.bio.indiana.edu/~ybelab/procedures/Chelating-Sepharose.pdf (accessed July 5, 2007). Maximum linear flow rate reported was 700 cm/h.

BM700515M