

FUNCTIONALIZED POROUS MEMBRANES FOR RAPID
PROTEIN PURIFICATION AND CONTROLLED
PROTEIN DIGESTION PRIOR TO
MASS SPECTROMETRY ANALYSIS

By

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ABSTRACT

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With the increasing demand for proteins in biotechnological research and applications, high-throughput protein purification is crucial. Convective mass transport and short radial diffusion distances in membrane pores make membrane absorbers attractive for rapid protein capture. However, the binding capacity of membranes is modest compared to bead-based techniques. Layer-by-layer polyelectrolyte adsorption should provide a simple way to functionalize membranes for extensive protein capture. Preliminary studies showed that poly(allylamine) (PAH)/poly(acrylic acid) (PAA) films adsorbed at pH 3 on Au-coated wafers swell up to 500% in pH 7 buffer. At neutral pH, (PAH/PAA)_n films are highly ionized and capture the equivalent of many monolayers of lysozyme without causing changes in film roughness.

Nylon membranes containing PAA/polyethyleneimine (PEI)/PAA films adsorbed at pH 3 provide a porous platform with a high density of –COOH groups that are available for further derivatization with NTA-Ni²⁺ complexes. When modified with PAA/PEI/PAA-NTA-Ni²⁺, membranes enable rapid isolation of His-tagged proteins from cell extracts with > 95% purity and recovery. Amazingly, the whole protein purification takes less than 30 min. A new membrane device developed by our commercial partners should require only 5 min to purify His-tagged protein from cell extracts. Finally, a polymer containing NTA as part of its side chain will avoid derivatization steps and greatly simplify the membrane-modification process.

Membrane adsorbers can also include immobilized proteins as their affinity agents. However, a membrane containing Concanavalin A (Con A, a protein that binds specific glycoproteins) did not provide a high glycoprotein binding capacity. Nevertheless, an antibody-containing membrane has the potential for rapid immunoaffinity purification of specific antigens with a binding capacity significantly higher than that of commercial resins.

Thin membranes also present an excellent platform for creating enzyme reactors. Covalent binding to PAA-modified membranes immobilizes about 35 mg of trypsin per cm³ of membrane pore, which is a 2800-fold increase in enzyme concentration relative to in-solution digestion. This high enzyme concentration also yields a 75-fold increase in digestion rate compared to typical in-solution methods. In addition, the covalent immobilization successfully prevents enzyme leaching and stabilizes the enzyme in 6 M urea or at pH 5, which aids digestion of proteins that require denaturation. Even after 6 months of storage at room temperature, the membrane can still digest protein in ms residence times. Moreover, thin (110 μm) membrane reactors afford fine control over proteolysis time and, hence, peptide lengths to enhance protein analysis. For β-casein digestion, msec digestion gives a large peptide that carries all five phosphorylation sites. Thus, such large peptides should enable correlation of post-translational protein modification events such as phosphorylation.

I dedicate this dissertation to my parents, Xinmin Dong and Junzhi Wu, and my brother, Yueqiang Dong, for their love and support.

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KEY TO ABBREVIATIONS

AAS	Atomic absorption spectroscopy
CAN	Acetonitrile
AEX	Anion-exchange
AG	α -D-allyl glucoside
Aminobutyl NTA	N_{α},N_{α} -Bis(carboxymethyl)-L-lysine
Asn	Asparagine
ATR-FTIR	Attenuated total reflectance fourier transform infrared
BA	N_{α} -benzoyl-L-arginine
BAEE	N_{α} -benzoyl-L-arginine ethyl ester hydrochloride
BSA	Bovine serum albumin
CSN8	COP 9 signalosome complex subunit 8
Con A	Concanavalin A
DEAE	Diethylaminoethanol
DMF	N,N -dimethylformamide
EDC	1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
FA	Formic acid
FPT	Freeze-pump-thaw
HEMA	2-hydroxyethyl methacrylate
His-tag	Histidine-tag

HisU	Histidine ₆ -tagged Ubiquitin
IgG	Immunoglobulin G
LAMA	2-lactobionamidoethyl methacrylate
LbL	Layer-by-layer
LCST	Lower critical solution temperature
Me ₆ TREN	Tris(2-(dimethylamino)ethyl)amine
MPA	3-mercaptopropionic acid
MS	Mass spectrometry
nESI	Nanoelectrospray ionization
NHS	N-Hydroxy succinimide
NTA	Nitrilotriacetate
PAA	Poly(acrylic acid)
PAH	Poly(allylamine)
PEI	Polyethyleneimine
PEMs	Polyelectrolyte multilayers
PET	Polyethylene terephthalate
PNTA	Poly(2,2-(5-acrylamido-1-carboxypentylazanediyl) diacetic acid)
Poly(MES)	Poly(2-(methacryloyloxy)ethyl succinate)
PSS	Poly(sodium styrene sulfonate)
PTMs	Post-translational modifications
PVAm	Poly(vinyl amine)
PVCL	Poly(N-vinylcaprolactam)

RC	Regenerated cellulose
RHD3	Root hair defective protein
RT	Room temperature
PAGE	Polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SI-ATRP	Surface initiated atom-transfer radical polymerization
TMSPA	Poly(3-(trimethyl) propargyl methacrylate)

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Chapter 1. Introduction and recent progress

This dissertation discusses development of functionalized porous membranes for protein purification and protein digestion prior to mass spectrometry (MS) analysis. When modified with polymeric films that swell in water, these membranes provide rapid Histidine-tagged (His-tagged) protein purification and immunopurification with binding capacities higher than those of commercial beads. The thin membrane also serves as a unique platform for enzyme reactors. Compared to in-solution digestion, trypsin-containing membrane reactors increase the rate of protein digestion and afford control over the length of proteolytic peptides. To put this work in perspective, I first introduce the advantages and development of membranes modified with polymeric thin films (section 1.1). Subsequent sections review recent progress in membrane-based protein purification via affinity interactions (section 1.2) and ion-exchange and hydrophobic interactions (section 1.3), phosphopeptide enrichment (section 1.4) and protease membrane reactors (section 1.5). A final section outlines this dissertation.

1.1. Membrane modification with polymeric thin films

1.1.1. Advantages of membranes

The current therapeutic protein market is nearly 100 billion U.S. dollars per year and growing rapidly (2). Because downstream purification and recovery account for almost half of the manufacturing costs for cell-derived pharmaceuticals (3-5), high-throughput and high-recovery separation techniques are essential in therapeutic protein production. Convenient, high-throughput purification is also important for fundamental protein research, especially with proteins that degrade rapidly, e.g. those in protease-containing solutions (6, 7).

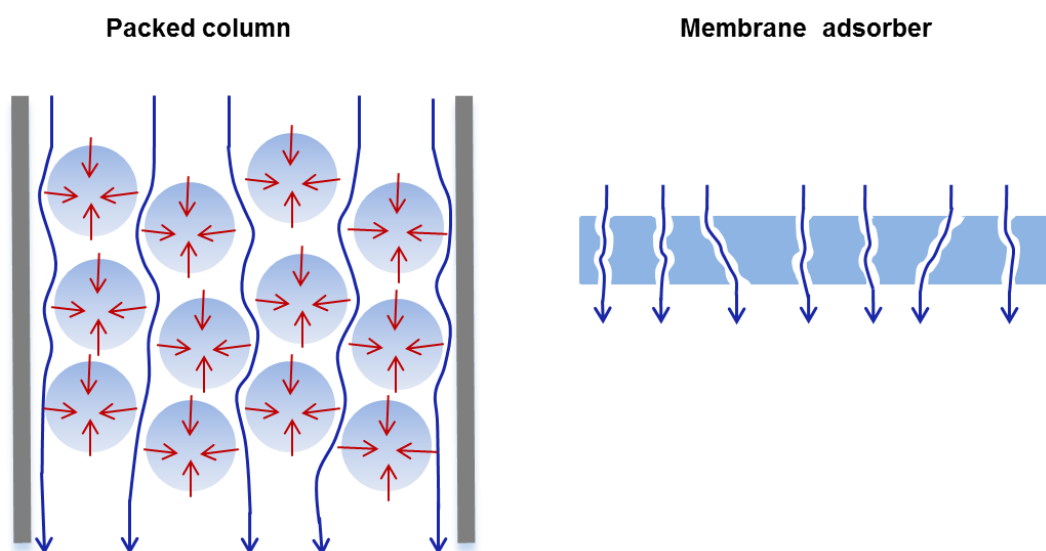


Figure 1.1. Transport phenomena involved in bead-based chromatography and membrane adsorbers. Blue arrows: convective flow; Red arrows: diffusion. The figures neglects film diffusion at pore and bead surfaces. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis (or dissertation).

Bead-based chromatography is the dominant technique in protein purification. Despite the success of this technique, slow diffusion of biomacromolecules into porous beads restricts throughput (8-11). Membrane-based methods should overcome diffusion

limitations to enhance rates of protein purification because convective flow passes through membrane pores and radial diffusion distances are small (**Figure 1.1**). Moreover, pressure drops across membranes with thicknesses around 100 μm are low, so high flow rates are easy to achieve (3). Thus, membrane adsorbers are a promising alternative to packed columns for rapid protein isolation (10, 12, 13).

Nevertheless, membranes historically suffered from modest binding capacities due to low specific surface areas relative to porous beads. This chapter highlights recent membrane functionalization strategies that achieve selective protein binding with capacities equal to or greater than those of porous beads. Moreover, protein immobilization techniques enable fabrication of enzyme-containing membranes that serve as proteolytic reactors with residence times as low as a few milliseconds (ms). In membrane-based protein digestion, control over residence time enables the formation of large proteolytic peptides that often facilitate protein characterization by MS. This review first describes membrane modification by coating pores with polymer brushes or polyelectrolyte multilayers (PEMs). Subsequent sections explore protein purification using metal-affinity adsorption, ion-exchange and multimodal interactions. Modification of membranes with functional polymeric films leads to highly selective protein capture with high binding capacities. Later sections show that membranes containing TiO_2 nanoparticles or proteases enhance MS studies of protein structure and phosphorylation. Finally, I summarize recent advances and highlight a few challenges for membrane applications.

1.1.2. Modification of membranes with functional polymeric thin films

A wide range of porous materials including alumina (14), silica (15) and many polymers (16) have served as membranes for protein purification. With all materials, however, selective binding relies on functionalization of the porous support with affinity groups. Moreover, high-capacity protein capture requires a 3-dimensional binding scaffold, i.e. a highly swollen polymer film, on the surface of membrane pores.

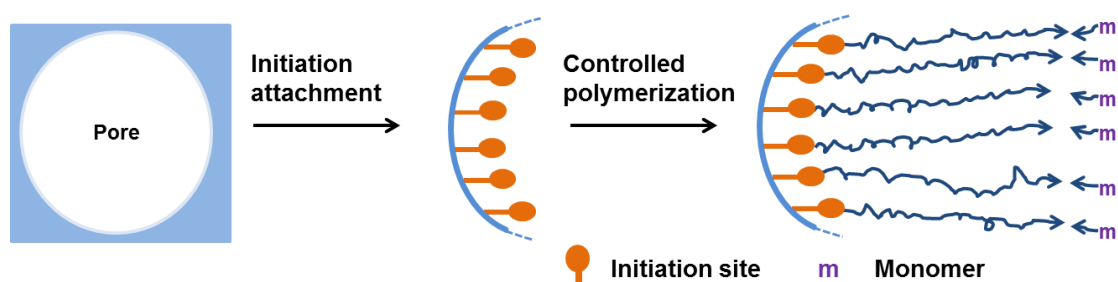


Figure 1.2. Schematic diagram of growth of polymer brushes from initiators attached to a membrane surface.

Polymer brushes, assemblies of polymer chains with one end attached to a substrate and one end extended from the surface (17), are attractive for binding multilayers of protein in membrane pores. Brush formation in membranes usually employs polymerization from surfaces to achieve high polymer-chain areal densities (10, 18, 19). Thus, the brush synthesis typically includes initiator attachment to the membrane and polymer growth from these immobilized initiators (**Figure 1.2**) (10, 19). Among many techniques for brush growth, surface initiated atom-transfer radical polymerization (SI-ATRP) is particularly useful. ATRP offers controlled polymerization of a wide range of monomers under mild conditions and uses readily available catalysts and initiators (20-23). Several groups modified a variety of membranes using ATRP from immobilized

initiators, and the binding capacities of such membranes often exceed 100 mg of protein per mL of membrane (24-28).

The amount of protein binding in polymer brushes varies with the polymer-chain areal density. Low chain densities give a small number of binding sites and minimal protein capture, whereas high densities may result in steric hindrance to protein entry into the brush. Hence, an intermediate chain areal density will likely lead to the most protein binding. Chain density depends in part on the density of initiation sites anchored to membrane surfaces, and anchoring typically occurs through surface functionalities such as hydroxyl groups (29) and carboxylic acids (30). However, some membranes have low densities of such surface functional groups. Jain and coworkers overcame this obstacle using LbL adsorption of a macroinitiator onto a polyethersulfone membrane and successfully modified the membrane using ATRP from the macroinitiator (24).

Several studies demonstrated control of chain densities in polymer brushes through dilution of initiators during their immobilization (27, 30-32). Reduced polymer-chain density decreases film thickness but increases the degree of swelling to allow protein penetration to binding sites (30, 31). Recently, Bhut and Husson controlled the grafting density of poly(2-(methacryloyloxy)ethyl)trimethylammonium chloride) in regenerated cellulose (RC) membranes by varying the ratios of ATRP-initiator and a non-ATRP-active diluent molecule during initiator immobilization (28). Membranes containing the highest density of polymer chains gave the highest IgG binding capacity, indicating minimally restricted IgG access to binding sites in this case. However, the protein-binding capacity did decrease at high polymer densities in commercial strong

cation-exchange resins (33).

1.2. Protein purification using affinity membranes

Among the strategies for protein purification, binding to immobilized affinity ligands is probably the most powerful (16). Ligands exploited for affinity capture include antibodies, protein A and protein G (IgG-binding proteins), lectins (a group of carbohydrate-binding proteins), carbohydrates (for lectin capture), and metal-ion complexes that capture His-tagged proteins or phosphopeptides (10). This section focuses on recent development of membranes that capture His-tagged proteins and lectins and then briefly examines the fabrication of affinity membranes through molecular imprinting

1.2.1. His-tagged protein purification

His-tags are oligo-histidine (commonly 6 residues) clusters fused to the N- or C-termini of recombinant proteins. These clusters form chelating complexes with immobilized Cu(II), Ni(II), Zn(II) or Co(II) during immobilized metal-ion affinity chromatography (34) and are the most common affinity tags for protein purification (35). Two reviews summarized early work on creating membranes for His-tagged protein purification (10, 19), so this section describes recent development of simpler but effective membrane-modification techniques.

Using SI-ATRP, Jain and coworkers grew poly(2-(methacryloyloxy)ethyl succinate) (poly(MES)) from nylon membranes previously modified by reaction with a trichlorosilane initiator (35). The aqueous MES polymerization is compatible with a wide

range of polymeric membrane supports. After derivatization of the –COOH groups on poly(MES) with aminobutyl NTA, formation of Cu^{2+} and Ni^{2+} complexes allows protein adsorption through metal-ion affinity. Modified membranes containing Cu^{2+} have a bovine serum albumin (BSA)-binding capacity of 80 mg/mL, whereas membranes with Ni^{2+} complexes capture 85 mg of His-tagged ubiquitin per mL of membrane. Remarkably, these binding capacities are about twice those of commercial metal-ion affinity resins (36). Moreover, the membrane modified with poly(MES)-NTA- Ni^{2+} isolated His-tagged cellular retinaldehyde-binding protein from *E. coli* cell lysates in only 10 min.

In the above-mentioned procedure, MES polymerization occurs in water but reaction of the membrane with the trichlorosilane initiator takes place in tetrahydrofuran, which is incompatible with some polymeric substrates. Anuraj and coworkers developed a completely aqueous membrane modification pathway that uses a macroinitiator (25). During 10-min of circulation through the membrane, the aqueous macroinitiator adsorbs to membrane pores via hydrophobic interactions. Remarkably, after only 5 min of subsequent MES polymerization from the macroinitiator, modified membranes attain protein-binding capacities as high as those after a 1-hour polymerization from membranes modified using a trichlorosilane initiator. Polymerization from the macroinitiator is a robust modification, as no significant drop in protein-binding capacity occurred after 5 cycles of protein binding and elution.

To clearly demonstrate the rapid protein binding that takes place in membranes, Anuraj and coworkers showed that the dynamic lysozyme-binding capacities of

poly(MES)-modified membranes do not decrease when increasing the flow through the membrane 30-fold, and binding occurs in residence times of only 35 ms. After reaction with aminobutyl NTA and formation of Ni^{2+} complexes, these membranes selectively capture His-tagged *myo*-inositol-1-phosphate synthase from a crude *E.coli* cell lysate. This aqueous modification procedure should apply to a wide range of porous membrane materials.

1.2.2. Lectin purification using glycosylated membranes

Carbohydrate-lectin interactions play important roles in biological processes such as cell–cell adhesion, signal transduction, tumor progression and host-pathogen recognition (37-42). Despite the importance and specificity of carbohydrate-protein interactions, however, the binding affinity is low ($K_a=10^3\text{-}10^4 \text{ M}^{-1}$) (43). Nevertheless, multiple interactions between lectins and dense sugar ligands overcome low affinity (44). The mechanism of multivalent interactions is not elucidated completely, but the so called “cluster glycoside effect” improved binding affinity in several studies (45-47). Thus, dense carbohydrates in membranes should bind lectins with high affinity, and convective mass transport will yield rapid capture.

Yang and coworkers recently grafted a sugar-containing monomer, α -D-allyl glucoside, onto a polypropylene membrane using UV-induced polymerization (**Figure 1.3a**). The membrane showed the “cluster glycoside effect”, as increasing the ligand density enhanced the binding capacity for a common lectin, Con A, with selectivity over contaminating BSA (44). Subsequently, the same group found that comblike

glycopolymers in membranes exhibit higher lectin binding than linear polymers. The comblike structure likely leads to both thicker films and higher densities of carbohydrates (**Figure 1.3b**). Membranes with a comblike glycopolymer containing galactose end groups captured 24 mg/mL of the lectin peanut agglutinin. Considering the ~ 10% porosity of the track-etched polyethylene terephthalate (PET) in that study, membranes with 50% porosity might capture 100 mg of lectin per mL of membrane (48). Additionally, the high density of sugar-containing chains provides resistance to nonspecific adsorption, and BSA did not bind significantly to the membrane.

Using a two-step grafting strategy, Wang and coworkers established a second route to modify polypropylene membranes with carbohydrates. After pretreatment, they grafted poly(3-(trimethyl) propargyl methacrylate) (TMSPA) to the membrane using UV-initiated graft polymerization. Subsequent “Click” coupling by reaction of 2,3,4,6-tetra-*o*-acetyl- β -D-glucopyranoside thiol with the alkyne groups on poly(3-(trimethyl) propargyl methacrylate) gave a high density of carbohydrate sites (**Figure 1.3c**). This sugar-containing polymer selectively binds Con A and resists capture of peanut agglutinin (49). These studies clearly show the promise of membrane adsorbers for lectin purification.

1.2.3. Molecularly imprinted polymer membranes

Molecular imprinting usually creates recognition sites through templating of a polymer scaffold (50). However, imprinting is challenging with macromolecules because of their flexible conformation (51). To overcome this limitation, Ulbricht and coworkers first created a poly(methacrylic acid) scaffold that adsorbs template proteins in track-etched PET membranes. After protein adsorption, UV-initiated cross-linking using polyacrylamide led to a cross-linked hydrogel, and the “cavities” left by template elution served as recognition sites for target proteins (**Figure 1.4**). Tailoring the polymerization and crosslinking eventually yielded a lysozyme:cytochrome C selectivity as high as 18 from a 1:1 mixture of these proteins, which have similar sizes and isoelectric points (52). They also developed an IgG-imprinted membrane to separate IgG from human serum albumin, a major contaminating protein in antibody downstream processing. The binding capacity for IgG was 8 mg/mL and the IgG/ human serum albumin selectivity was 5 (53). Further improvements in binding capacity and selectivity are needed to make separations with imprinted membranes competitive with current techniques.

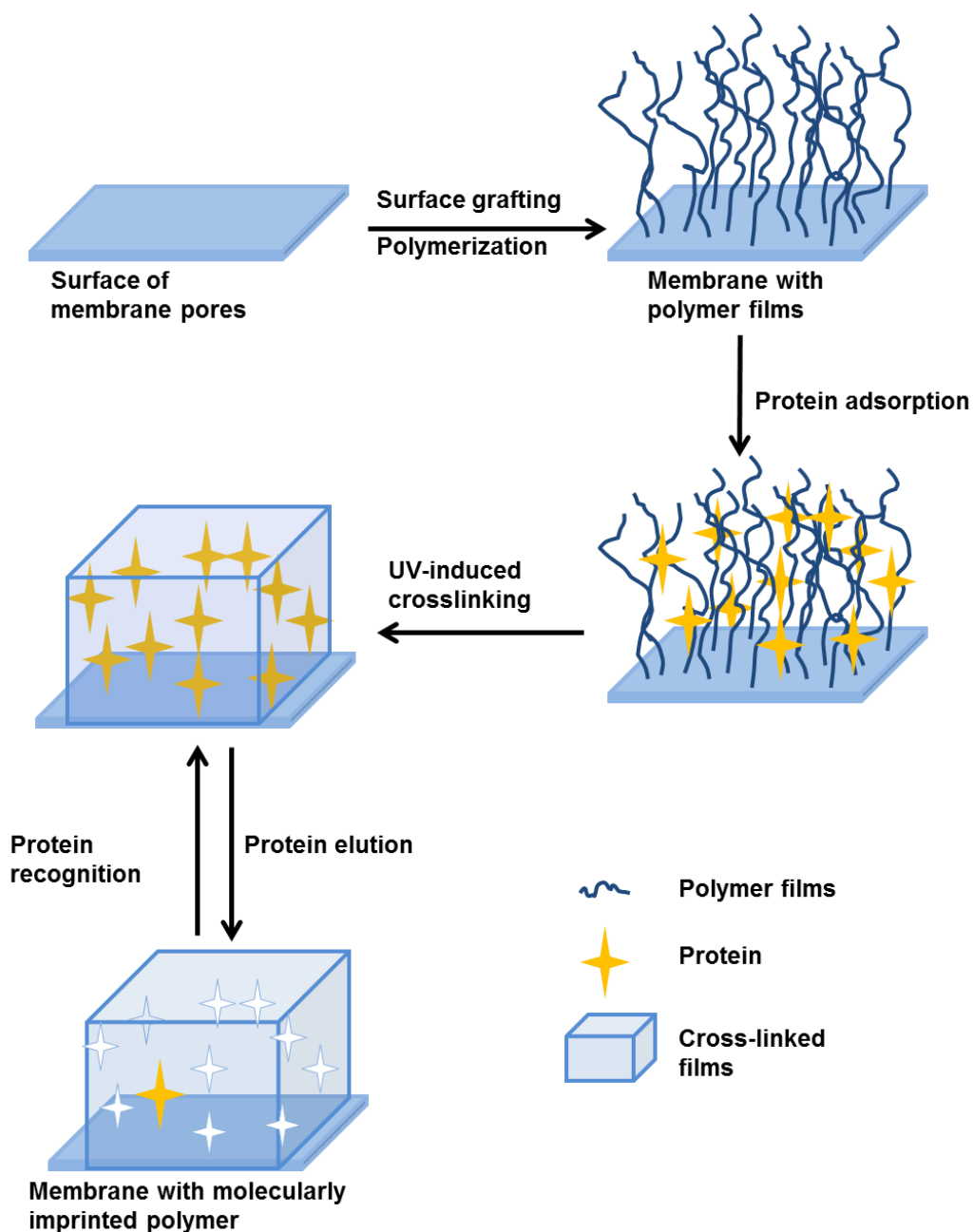


Figure 1.4. Protein imprinting via two step grafting using SI-ATRP and UV-initiated grafting/crosslinking copolymerization on the membrane surfaces.

1.3. Protein purification via ion-exchange and hydrophobic interactions

In addition to affinity methods, protein isolation with membrane adsorbers can occur through less specific ion-exchange and hydrophobic interactions or their combination. The subsections below describe recent advances in these areas.

1.3.1. Ion-exchange membranes for protein capture

Ion-exchange protein separations rely on electrostatic interactions between charged moieties on membranes, e.g. carboxylate (54), sulfonate (55) and ammonium (5) groups, and surface charges on biomolecules. Although several companies provide ion-exchange membranes (16), the materials have relatively low binding capacities (<50 mg of lysozyme/mL of membrane) (56). Thus, several research groups aimed to improve the protein-binding capacity of ion-exchange membranes while maintaining reasonable membrane permeability.

Wang and coworkers prepared a cation-exchange membrane through UV-initiated grafting of poly (acrylic acid (PAA) and PAA co-polymers on macroporous RC membranes. These membranes showed dynamic binding capacities (protein adsorbed at the point of 10% break through) of 90 mg of lysozyme per mL of membrane and 66 mg of IgG per mL of membrane (54). Subsequently, Chenette and coworkers developed strong cation-exchange RC membranes via organic solvent-based SI-ATRP of 3-sulfopropyl methacrylate (57). Protein purification with sulfonates avoids potential challenges with incomplete dissociation of carboxylate groups. The poly(3-sulfopropyl methacrylate)-modified membranes exhibited a dynamic lysozyme binding capacity as high as 70 mg/mL at a throughput of 100 column volumes/min and a productivity of 300 mg/mL/min whereas the productivity of a commercial Capto S resin column was only 4 mg/mL/min. Wei and coworkers fabricated a high-capacity strong cation-exchange membrane using SI-ATRP of poly(sodium 4-styrenesulfonate) on RC membranes (55). The controlled polymerization reduced the membrane roughness and provided a

lysozyme static binding capacity of 138 mg/mL and a binding capacity of 93 mg/mL at 50% break-through.

A number of studies reported new methods for fabrication of anion-exchange (AEX) membranes (5, 13, 27, 28). Bhut and coworkers used SI-ATRP to create poly (2-dimethylaminoethyl methacrylate) nanolayers on RC membranes. The static BSA binding capacity was 66 mg/mL (5). They dramatically improved the performance of their membranes by optimizing the initiator density and poly (2-dimethylaminoethyl methacrylate) molecular weight to achieve a BSA binding capacity of 130 mg/mL at a linear flow velocity >350 cm/h (27). The binding capacity was >35 mg/mL even with 100 mM ionic strength, and BSA isolation from a protein mixture occurred with >98% recovery and >97 % purity.

Compared to commercial AEX Sartobind®D membranes and fast flow columns prepacked with diethylaminoethanol (DEAE) Sepharose beads (HiTrap™ DEAE FF resins), Bhut's AEX brush membranes showed superior performance. The breakthrough curves of the HiTrap™ DEAE FF resin columns changed at different flow rates, presumably because diffusion of BSA to the capture sites is not fast enough for binding to keep up with convection. Even worse, binding capacities decreased as flow rate increased. In contrast, for the Sartobind®D membrane and the new AEX membranes, the breakthrough curves and binding capacity were independent of flow rate because convective flow dominates the mass transport and the kinetics of binding are rapid (13). Notably, the AEX brush-modified membrane shows a binding capacity 3 times that for the Sartobind®D membrane. Bhut and coworkers also developed a strong AEX

membrane containing quaternary amines (Q-type AEX membrane) (28). By controlling the graft density, the Q-type AEX membrane showed a flow-rate independent dynamic IgG binding capacity of greater than 130 mg/mL, enabling high throughput protein purification. Clearly, brush-modified membranes can greatly increase the rate of ion-exchange protein separations, while enhancing binding capacity 2- to 3-fold compared to commercial ion-exchange systems.

1.3.2. Hydrophobic and multimodal protein purification membranes

Most protein-binding membranes have hydrophilic surfaces to increase pore wetting with water and minimize non-specific binding and membrane fouling (58, 59). However, interactions between hydrophobic surface patches on proteins and hydrophobic ligands on a solid support can also effect protein separations (16, 60). Hydrophobic chromatography usually relies on protein loading from solutions with high concentrations of salts and subsequent elution with solutions of decreasing ionic strength (61, 62). Recently, Himstedt and Wickramasinghe used SI-ATRP to graft poly(*N*-vinylcaprolactam) (PVCL) from RC and create membranes for hydrophobic chromatography (63). PVCL is a thermo-responsive polymer. At temperatures above the lower critical solution temperature (LCST) the polymer loses water and collapses, and the LCST of PVCL decreases with increasing ionic strength and moves below room temperature in buffers containing 1.8 M $(\text{NH}_4)_2\text{SO}_4$. Thus, in solutions with >1.8 M $(\text{NH}_4)_2\text{SO}_4$, PVCL assumes a collapsed conformation, which is favorable for hydrophobic protein binding. Subsequent elution can occur at lower ionic strength where

the LCST is greater than room temperature. PVCL-modified membranes achieved a BSA binding capacity of 5 mg/mL, which is higher than that with non-modified PVDF membranes and comparable to Sartorius hydrophobic membranes. The IgG binding capacity is 21 mg/mL or about twice that of Sartorius membranes (64, 65).

To improve membrane performance, Wang and Husson developed a multimodal cation-exchange membrane that employs both Coulombic and hydrophobic interactions (66). They grafted poly(glycidyl methacrylate) from RC membranes and then coupled 4-mercaptobenzoic acid to the polymer side chains. At pH 6.5 without salt, these membranes bind 150 mg IgG /mL of membrane in static measurements. Remarkably, the membranes retain 90% of their static binding capacity in the presence of 1 M sodium citrate, a kosmotropic salt found in elution buffers for protein A chromatography. However, effective use of these multimodal membranes will require development of an efficient elution strategy. In addition, protein binding and selectivity in dynamic modes need more study.

1.4. Phosphopeptide enrichment with TiO₂-containing membranes

Adsorption of nanoparticles provides another option for introducing selective binding sites into membranes. Specifically, Tan and coworkers modified membranes with TiO₂ nanoparticles to capture the phosphopeptides in protein digests (**Figure 1.5**). Protein phosphorylation on serine, threonine or tyrosine amino acid residues is one of the most important mechanisms for creating protein diversity in eukaryotic cells, and regulation of protein phosphorylation affects nearly all aspects of cell life, including signal

transduction and metabolism. Abnormal phosphorylation can cause diseases including cancer (67-69), so successful analysis of phosphorylated proteins is vital for understanding disease stages and discovering potential pharmaceutical treatment targets (70, 71).

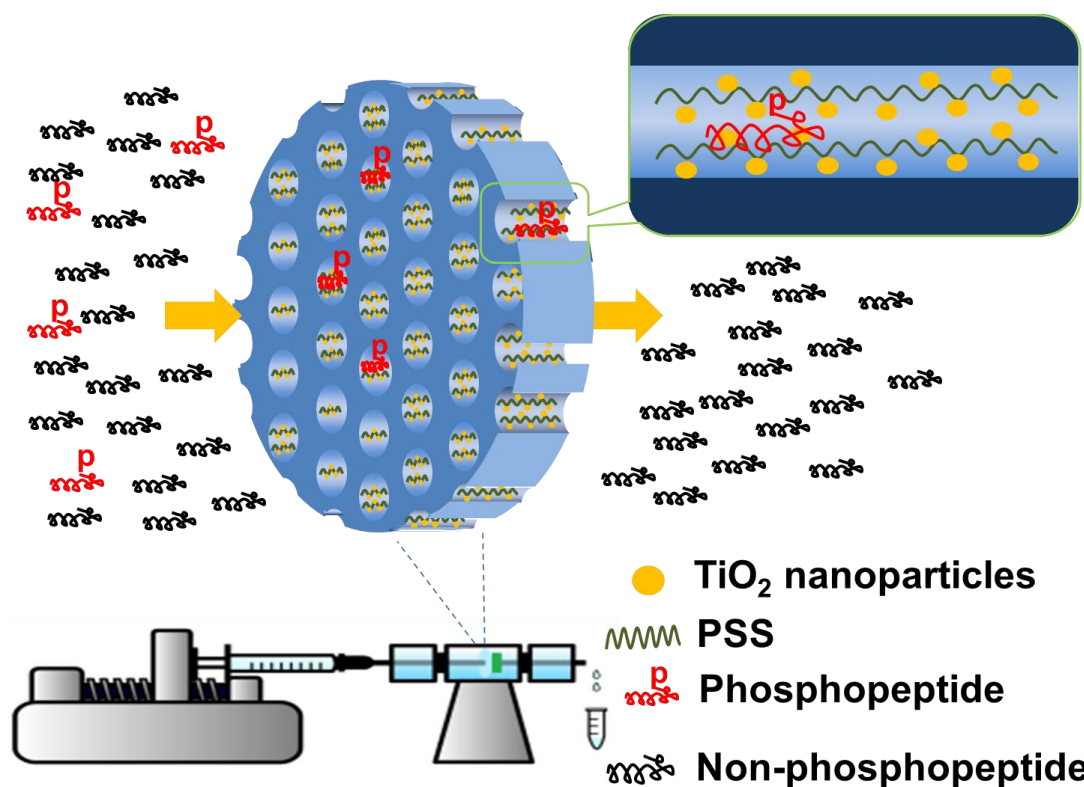


Figure 1.5. Cartoon of selective phosphopeptide capture in a membrane containing TiO_2 nanoparticles. A small holder attached to a syringe pump enables phosphopeptide elution in as little as 10 μL of solution. Adapted from reference 76 with permission of the American Chemical Society.

Because of the low relative abundance of phosphorylated proteins, the detection and identification of phosphorylation sites is challenging even with recent advances in MS (72). However, a number of selective phosphopeptide enrichment methods help to overcome this challenge (19, 73, 74). In particular, phosphopeptide capture can occur

through selective adsorption on TiO₂ or ZrO₂ columns or on MALDI plates containing TiO₂ nanoparticles (75, 76). Membranes are attractive for immobilizing TiO₂ nanoparticles that are too small for column formats, and such particles have high surface areas for phosphopeptide capture and may show different selectivities than larger particles.

Tan and coworkers modified nylon membranes by sequentially adsorbing poly(sodium styrene sulfonate) (PSS) and TiO₂ nanoparticles in membrane pores (77). Negatively charged sulfonate groups bind the positively charged nanoparticles, and energy-dispersive X-ray spectroscopy shows a uniform Ti coverage over the whole cross-section of the membrane. With 22-mm-diameter membranes adsorption of two PSS/TiO₂ bilayers results in 55 μ mol of Ti per membrane, and the immobilized TiO₂ binds 540 nmol (12 mg/mL) of phosphoangiotensin when saturated. This capacity is significantly lower than the protein-binding capacities of polymer brush-modified membranes because PSS/TiO₂ nanoparticle adsorption does not give thick, highly swollen films. Notably, however, the TiO₂-containing membranes show 90% phosphoangiotensin recoveries even after 10 cycles of loading, washing and elution. In competition experiments, the phosphopeptide recovery is >70% in a 15-fold excess of BSA.

Membranes with working areas of only 0.02 cm² can isolate phosphopeptides from solution volumes ranging from a few mL to 10's of μ L. For example, Tan and coworkers selectively captured phosphopeptides from 5 mL of a 1 fmol/ μ L protein digest and concentrated these peptides into elution volumes of 10's of μ L. Additionally, they

enriched phosphopeptides from a digest of hyperphosphorylated tau (p-tau), a protein whose abundance correlates well with the severity of neurodegeneration in Alzheimer's disease. Before enrichment, MALDI-MS analysis of the digest gave 63 p-tau peptide signals with intensities >10% of the highest signal, but none of these peptides were phosphorylated. In contrast, after enrichment using the TiO₂-containing membrane, signals from four phosphopeptides appeared, one of which showed the highest intensity in the MALDI mass spectrum. The enrichment led to identification of 7 phosphorylation sites in MS/MS analysis.

1.5. Protease-containing membranes for controlled protein digestion prior to MS analysis

With methods in hand to immobilize proteins in membranes (see sections 1.2 and 1.3), the Bruening group began employing enzyme-modified membranes as controlled reactors for protein digestion prior to MS analysis. MS is the most common and powerful technique for identifying proteins and their posttranslational modifications (78). Nevertheless, because peptides are more amenable to MS and Liquid Chromatography-MS analysis than proteins, proteolytic digestion is usually a vital initial step for MS protein analysis. Conventionally, digestion occurs after mixing a protease such as trypsin with substrate proteins in solution. However, this strategy requires low enzyme concentrations to restrict self-digestion of the protease, so digestion times are generally 1 hour or more (79). To overcome this drawback and facilitate online MS analysis, several research groups developed reactors with proteases immobilized on solid supports including monoliths (80, 81), membranes (82, 83), polymeric microfluidic

channels (82, 84) and resins (85, 86). With thicknesses of only 10-200 μm , membranes provide a unique platform for controlling protein digestion.

Xu and coworkers formed membrane reactors by sequential adsorption of PSS and trypsin in nylon membranes (87). Adsorption occurred at pH 3, where trypsin is positively charged and inactive, whereas digestion took place in 10 mM NH_4HCO_3 to optimize trypsin activity (88). This simple adsorption procedure immobilizes 10 mg of trypsin per mL of membrane pores, which is 450 times the enzyme concentration during typical digestion in solution. Moreover, the immobilized trypsin rapidly digests protein. After digestion of α -casein, gel electrophoresis showed no bands for intact protein even at membranes residence times as short as 0.79 s, indicating digestion of at least 93% of the protein. With a 3.2-s residence time, the MALDI-MS sequence coverages of the α -S1 and α -S2 chain were 53 % and 43%, respectively. In contrast after a 16-hour in-solution digestion, the sequence coverages were only 46% and 26%, respectively. Thus, in much shorter time, the trypsin membrane reactor provided equal or better digestion than the conventional in-solution digestion. Membranes were active during continual digestion for 33 h, and their shelf life is several months.

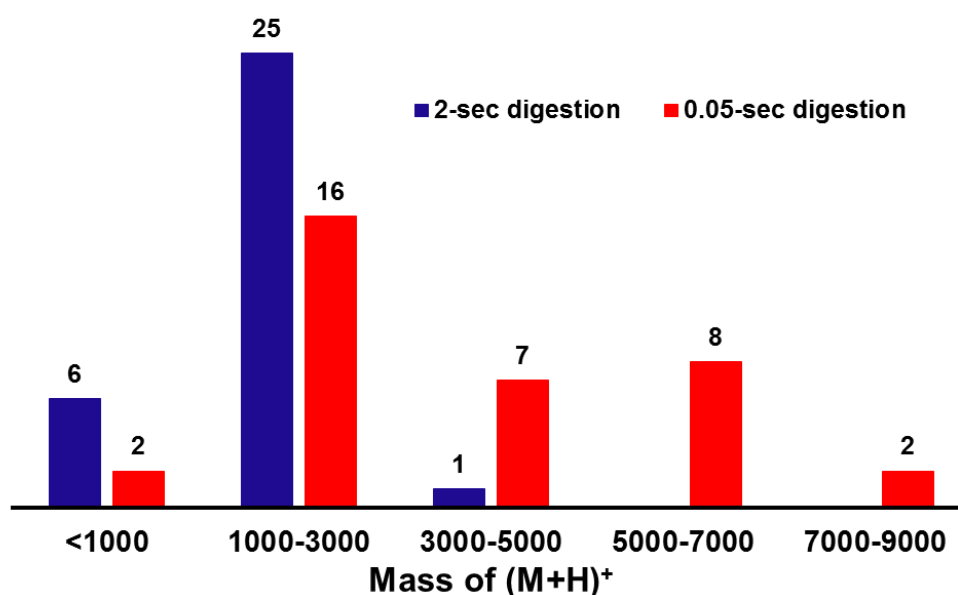


Figure 1.6. Peptide size distributions in ESI-Orbitrap mass spectra of BSA digested using a pepsin-containing membrane and residence times of 2 (blue) and 0.05 (red) s. The numbers above the bars are the number of recognized peptides in that mass range. Adapted from reference 88 with permission of the American Chemical Society.

Perhaps the biggest asset of membrane digestion is the control of peptide size afforded by variation of residence times down to the ms level. Short residence times should give large peptides due to missed cleavage sites. Tan and coworkers (89) reported limited proteolysis using membranes modified with PSS/pepsin coatings. Apomyoglobin digestion at pH 2 showed increasing peptide sizes with decreasing residence times. The controlled digestion improved protein sequence coverage in ESI-Orbitrap MS analysis. In limited digestion of denatured BSA, which contains 583 amino acid residues and 17 disulfide bonds, a 0.05-s resident time in a pepsin-containing membrane yielded 82% sequence coverage in ESI-Orbitrap MS analysis, whereas a 2-s residence time (a

complete digestion) gave only 53% sequence coverage. Complete digestion likely gives small peptides that are sometimes difficult to detect. **Figure 1.6** shows the peptide size distributions and clearly demonstrates that the 0.05-s digestion leads to larger peptides than digestion for 2 s. Larger peptides should enhance detection of posttranslational modifications because of greater sequence coverage.

Tan and coworkers employed limited digestion in PSS/trypsin-containing membranes to examine the structure of Root Hair Defective (RHD3) protein (89). RHD3 participates in fusion of the endoplasmic reticulum membrane and is essential in the growth of *Arabidopsis thaliana* (90), but with a complex structure including a GTPase domain, a helix bundle, two trans-membrane helices and one cytosolic tail, the biological roles and structure of RHD3 are difficult to interrogate. Tan and coworkers employed low membrane residence times to determine which sites are most accessible to digestion. Non-denatured RHD 3 was passed through a PSS/trypsin-modified membrane with residence times of 0.045, 0.2 and 1 s. Analysis of the digests by MS revealed the strongest signal for the peptide from amino acid residues 673-691 (RHD 3 has 691 total amino acid residues) for all residence times. Signals for other peptides showed a relative increase in intensity as digestion time increased. These results suggest that R672 is in the most flexible region of RHD 3, and truncating the protein at R672 may lead to crystallization.

1.6. Outline of the dissertation

The literature reviewed above clearly shows the potential of membranes to simplify

and enhance the rate of protein purification and also to control protein digestion. This dissertation first aims to develop new methods to more simply and inexpensively modify porous membranes for protein purification. In a second aim, the research examines covalent trypsin immobilization to stabilize membranes for protein digestion. To simplify membrane modification, we employ layer-by-layer polyelectrolyte deposition (LbL), which simply involves passing polyelectrolyte solutions through a membrane. Chapter 2 of this dissertation describes fundamental studies of LbL polyelectrolyte film properties on flat surfaces mainly using ellipsometry and reflectance FT-IR. With control over the growth conditions, these films swell extensively in water and capture multiple-layer of protein to provide high protein binding capacity. However, protein binding depends on pH, ionic strength and binding kinetics. This chapter examines the factors that affect protein binding LbL films.

In chapter 3, I adsorb PAA/polyethyleneimine (PEI)/PAA films in porous membranes and subsequently derivatize these films to capture His-tagged protein. His-tagged proteins bind to Ni^{2+} that is bound to NTA groups appended to the PAA/PEI/PAA films. This chapter investigates both metal-ion binding and protein binding and compares the recovery and specificity of protein capture to purification with commercial resins. To simplify the membrane fabrication process and decrease cost, Salinda Wijeratne of our group developed new polymers that I used to modify the membranes for his-tagged protein purification.

Membrane-based protein purification is not limited to His-tagged proteins. In chapter 4, I describe efforts to create glycoprotein purification membranes. Purification

of glycoprotein relies on interactions with lectins such as Con A. Thus, I immobilized Con A on the membranes using covalent or metal-affinity methods. The immobilization employed membranes containing polymer brushes or LbL films, but unfortunately glycoprotein binding was not extensive.

As reviewed above, thin membranes are unique supports for enzyme reactors because they afford control over reaction times down to the ms level. Chapter 5 describes the development and application of a reaction containing covalently immobilized trypsin. The covalent linkage prevents enzyme leaching and provides a high enzyme concentration that leads to digestion that is much faster than conventional in-solution methods. Moreover, these membranes afford control of peptide lengths by variation of the flow rate through the membrane. These reactors are more stable than membrane containing electrostatically adsorbed trypsin, and this stability enables digestion in harsh conditions such as 6 M urea. Digestion under such heavily denaturing conditions is not possible with in-solution methods.

Finally, chapter 6 summarizes the major findings in this work and highlight future applications.

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Chapter 2. Characterization of polyelectrolyte films used for protein capture

The long-range goal of this research is the development of coatings that conveniently capture proteins in porous membranes. Layer-by-layer (LbL) adsorption of polyelectrolytes is a particularly convenient method for preparing such coatings. This chapter describes fundamental studies of the growth, swelling, ionization, stability, and lysozyme binding of protonated poly(allylamine) (PAH)/poly(acrylic acid) (PAA) films on Au-coated Si wafers. Deposition of these films on flat wafers enables characterization using ellipsometry and reflectance FT-IR spectroscopy, which is not possible with films in membranes pores. With control over adsorption conditions, (PAH/PAA)_n films formed at low pH swell extensively in pH 7.4 buffers and capture multiple layers of lysozyme. However, lysozyme sorption occurs via ion exchange and thus depends on pH and ionic strength.

2.1. Introduction

Despite the successful application of polymer brushes to membrane functionalization, brush synthesis is complicated because it relies on initiator attachment and polymerization under anaerobic conditions (10). LbL adsorption of functional

polyelectrolytes, exposing a surface to alternating aqueous polyanion and polycation solutions with a water rinse between each exposure, provides a much simpler method to coat substrates (91, 92). The LbL method is essentially a multi-point “grafting to” technique, but sequential adsorption steps, judicious polyelectrolyte selection, and control over deposition conditions can tune polyelectrolyte multilayers (PEMs) for different purposes (93-95).

Since its introduction by Decher and Hong, LbL polyelectrolyte adsorption has emerged as one of the simplest and most versatile coating methods as it applies to a range of materials and geometries (96-98), including flat surfaces (91, 96), nanoparticles (99) and membranes (100, 101). Over the last 20 years, research groups have applied PEMs in sensors (102-105), biomimetic material (106-108), tissue engineering (109, 110) and drug delivery (111-113). Building blocks of PEMs can include polymers (91, 114-117), viruses (118, 119), enzymes (120, 121) and inorganic particles (96, 122, 123). The PEMs can also serve as a “reservoir” for biomolecules and preserve their bioactivity (92). For example, Jewell and coworkers incorporated DNA, RNA and nucleic acid-based agents in PEMs for drug delivery purposes (124).

My research employs PEMs for protein capture, and extensive protein sorption will likely require films that swell extensively in water. Variation of deposition parameters such as ionic strength and pH allows tuning of PEM ionization and swelling to control protein sorption (125). For example, Dragan and coworkers reported sorption of the equivalent of 30 nm of human serum albumin in cross-linked PEMs (126). They generated the PEMs by selectively cross-linking the poly(vinyl amine) (PVAm) layers in

(PVAm/ PAA)_n thin films and subsequently removing the PAA layer. This chapter aims at developing PEMs that adsorb the equivalent of many monolayers of protein without post-construction treatment, with an eventual goal of providing high protein binding capacity in modified membranes. Specifically, I focus on (PAH/PAA)_n films because under physiological condition PAA-containing PEMs show minimal nonspecific binding and their –COOH groups are readily available for derivatization with protein binding ligands (93, 127). Control over deposition conditions also leads to (PAA/PAH)_n films that capture large amounts of protein. This chapter characterizes the growth of (PAA/PAH)_n coatings on flat substrates as well as their swelling, stability, ionization, and lysozyme sorption. Later chapters investigate derivatization of these films to specifically capture His-tagged proteins, glycoproteins, and antigens both on flat surfaces and in membranes.

2.2. Experimental section

2.2.1. Materials

Poly(allylamine hydrochloride) (PAH, molecular weight 120,000 - 200,000 Da) was purchased from Alfa Aesar, and poly(acrylic acid) was obtained from Polysciences (molecular weight 90000 Da, 25 wt% solution in water) or Sigma-Aldrich (molecular weight 100000 Da, 35 wt% solution in water). Aqueous solutions of 0.02 M PAH and 0.01 M PAA were prepared in deionized water (18.2 MΩcm, Milli-Q) with 0.5 M aqueous NaCl, and solution pH values were adjusted by dropwise addition of 0.1 M NaOH or HCl. (Polymer concentrations are given with respect to the repeating unit.)

Lysozyme from egg white, sodium phosphate and sodium phosphate dibasic were purchased from Sigma-Aldrich and used without further purification. Au-coated silicon wafers (200 nm of sputtered Au on 20 nm of Cr on Si (100) wafers) were cleaned in a UV/O₃ chamber for 15 min prior to use.

2.2.2. Preparation of PEMs

Au-coated Si substrates (24 mm × 11 mm) were immersed in 5 mM 3-mercaptopropionic acid (MPA) in ethanol for 2 h, rinsed with ethanol and dried with N₂ to form a monolayer of MPA and create a negatively charged surface. These substrates were immersed in 0.02 M aqueous PAH (with 0.5 M NaCl, adjusted to the desired pH) for 5 min and subsequently rinsed with 10 mL of deionized water and blown dry with N₂. Substrates were then immersed in a polyanion-containing solution (0.02 M PAA with 0.5 M NaCl, adjusted to the desired pH value) for 5 min followed by the same rinsing and drying procedures. The process was repeated to form multilayer films.

2.2.3. Characterization of PEMs

“Dry” film thicknesses were determined using a spectroscopic ellipsometer (J.A. Woollam M-44) assuming a film refractive index of 1.5. Fitting the refractive index did not significantly alter the calculated thickness. In situ ellipsometry in aqueous solutions was performed using a home-built cell described previously (128, 129). In this case, both refractive index and thickness were fitting parameters. Reflectance FTIR spectra were obtained with a Thermo Nicolet 6700 FTIR spectrometer using a Pike grazing angle (80°) apparatus. A UV/ozone-cleaned gold wafer served as a background. Atomic force

microscope (AFM) images were acquired using a NanoScope IIIa scanning probe microscope (Veeco Instruments, CA) operating in the contact mode. Pyramidal-shaped Si_3N_4 tips, mounted on gold cantilevers (100 μm legs, 0.38 N/m spring constant) were used in air.

2.2.4. Determination of the average pK_a for PAA

The average pK_a value for PAA in aqueous solution was calculated from titrations with 0.1 M HCl. The polyelectrolyte was dissolved in aqueous NaOH (pH ~12), and the pH was adjusted to 10 before titration. For films, pK_a values for PAA were estimated based on the ratio of $-\text{COOH}$ and $-\text{COO}^-$ absorbances (peak heights) in FTIR spectra (1).

2.3. Results and discussion

Protein binding likely varies with film thickness and ionization. Thus, the following two subsections present ellipsometric studies of film thickness as a function the number of adsorbed layers and reflectance FTIR spectra that investigate the extent of ionization. Subsequent sections examine lysozyme binding and film stability.

2.3.1. Formation of (PAH/PAA)_n films

The driving force for PEM formation includes electrostatic interactions between polycations and polyanions and more importantly release of counterions to the solution to increase entropy (42, 43). With strong polyelectrolytes, addition of supporting electrolyte to deposition solutions (44) and temperature affect polyelectrolyte conformations and film thickness (45). In the case of weak polyelectrolytes such as PAH

and PAA, the pH of the deposition solutions also influences polyelectrolyte conformation and film growth (7, 46). Typically, adsorption pH values that give a low degree of ionization of a weak polyelectrolyte lead to thick films (7, 47). Because thick films are attractive for high protein sorption, I studied the adsorption of $(\text{PAH/PAA})_n$ films from polyelectrolyte solutions at pH 3, where PAA is only partially deprotonated, and at pH 9, where PAH is only partly protonated. Shiratori and Rubner performed a similar study, but they employed polyelectrolyte solutions with no added salt (5). Because Ma and other researchers found that the addition of 0.5 M NaCl to PAA and PAH deposition solutions greatly increases film thickness, this work examines adsorption from solutions containing 0.5 M NaCl (46, 48, 49).

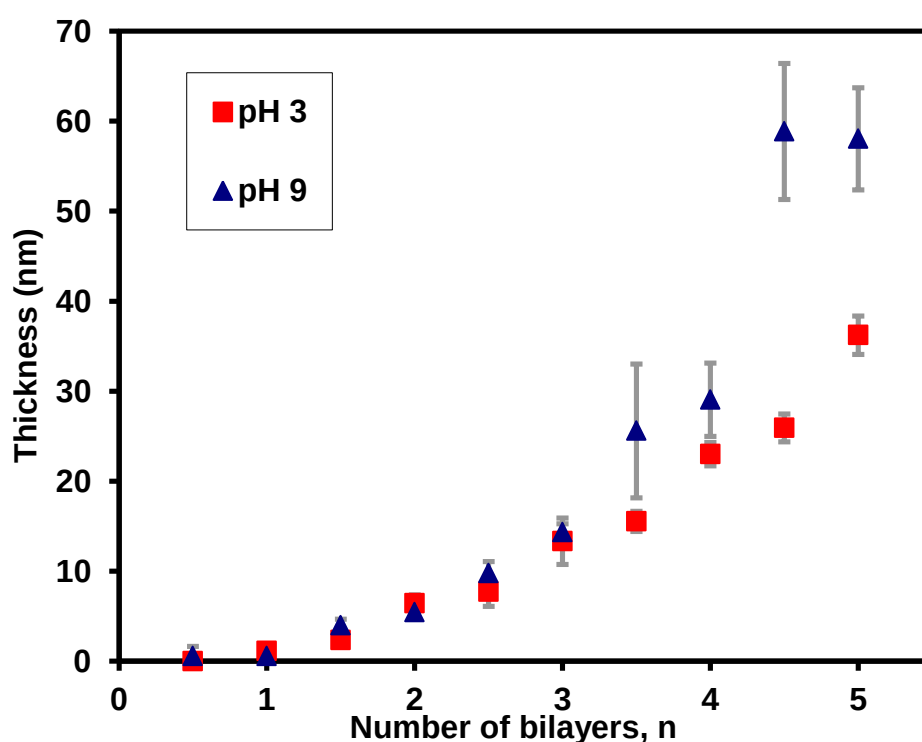


Figure 2.1. Ellipsometric thicknesses of $(\text{PAH/PAA})_n$ films deposited from 20 mM polyelectrolyte solutions containing 0.5 M NaCl at pH 3 (red squares) or pH 9 (blue triangles). When n is an integer, film deposition ends after PAA adsorption, whereas noninteger values indicate termination with PAH.

Figure 2.1 shows the growth of (PAH/PAA)_n films with the number of adsorption steps at pH 3 and pH 9. The total film thickness is similar for the two deposition pH values over the first 3 bilayers. However, for additional bilayers, films deposited at pH 9 show higher thicknesses. At pH 3, PAA is mostly protonated and contains a high density of –COOH in its structure. As mentioned above, the low charge density on the polymer at low pH likely gives a compact, rather than extended, polymer chain and this results in thicker films compared to adsorption of highly ionized PAA. Similarly, at pH 9 deprotonation of PAH leads to a low charge density and compact conformation of this polyelectrolyte. As a result, deposition at either pH 3 or 9 leads to relatively thick films compared to adsorption at neutral pH where film thickness is less than 10 nm for 5 bilayers (130).

2.3.2. PAA ionization in (PAH/PAA)_n films

Adsorption of PAA in (PAA/PAH)_n films changes its pK_a value. Based on the titration curve in **Figure 2.2**, the average pK_a of free PAA in solution is ~ 6.3. For this titration, the PAA solution was adjusted to pH 10 where we assumed all the –COOH groups were deprotonated, and titration with 0.1 M HCl followed. Equation (2.1) describes the degree of ionization, where COO[–] and COOH represent the moles of deprotonated and protonated acid groups, respectively.

$$\alpha = \frac{COO^-}{COO^- + COOH} \times 100\% \quad (2.1)$$

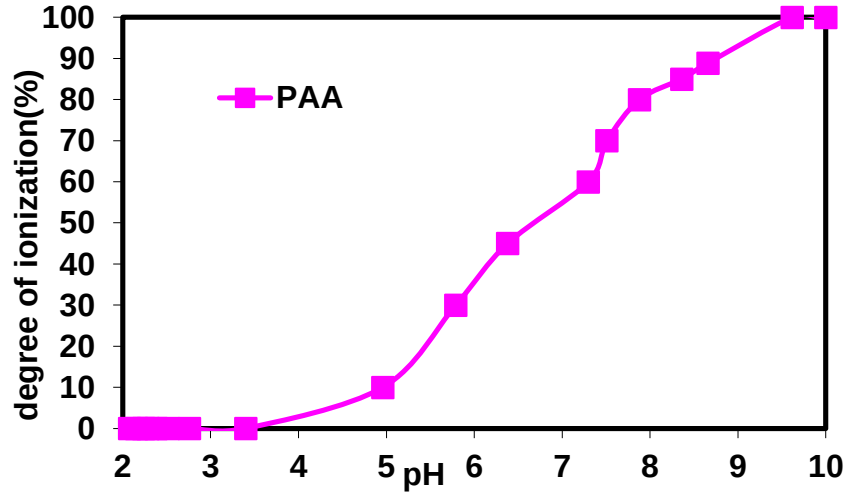


Figure 2.2. Titration of deprotonated PAA with 0.1 M HCl. The number of $-\text{COOH}$ groups at a given pH value was determined with the following equation, assuming that all the $-\text{COOH}$ groups were deprotonated at pH 10.

$$H^+ \text{ added} = \text{COOH} + \text{OH}^- \text{ neutralized} + \text{increase in free } H^+ \text{ in solution.}$$

The titration curve for PAA is similar to that in the literature (1). The text defines the degree of ionization.

To estimate the pK_a of the PAA in $(\text{PAA/PAH})_n$ films, we employed reflectance FT-IR spectroscopy (**Figure 2.3**). With the assumption of the same extinction coefficients for acid carbonyl and carboxylate absorption bands (1), equation (2.2) gives the degree of ionization in the film.

$$\alpha = \frac{A_{\text{COO}^-}}{A_{\text{COO}^-} + A_{\text{COOH}}} \times 100\% \quad (2.2)$$

In this equation, A_{COO^-} is the maximum absorbance of the carboxylate band ($\sim 1580 \text{ cm}^{-1}$) and A_{COOH} is the maximum absorbance of the acid carbonyl band ($\sim 1720 \text{ cm}^{-1}$).

We obtained values for the degree of ionization by immersing $(\text{PAH/PAA})_5$ films in

different buffers and subsequently acquiring their reflectance FTIR spectra. Based on equation (2.2) and the data in **Figure 2.3**, the estimated pK_a value of the $-COOH$ groups in PAA is 5.3 (the pH at which $A_{COO^-} = A_{COOH}$), which is about 1-unit lower than the pK_a value of the polymer in solution. Note that the pK_a value for PAA is an average value, and that removal of protons becomes more difficult as the polymer reaches an increasing degree of ionization. Thus the average pK_a value for PAA in solution is about 2 units higher than the pK_a value of acetic acid. In the film, electrostatic interactions with neighboring ammonium groups likely decrease the energy of required to form $-COO^-$ groups (130).

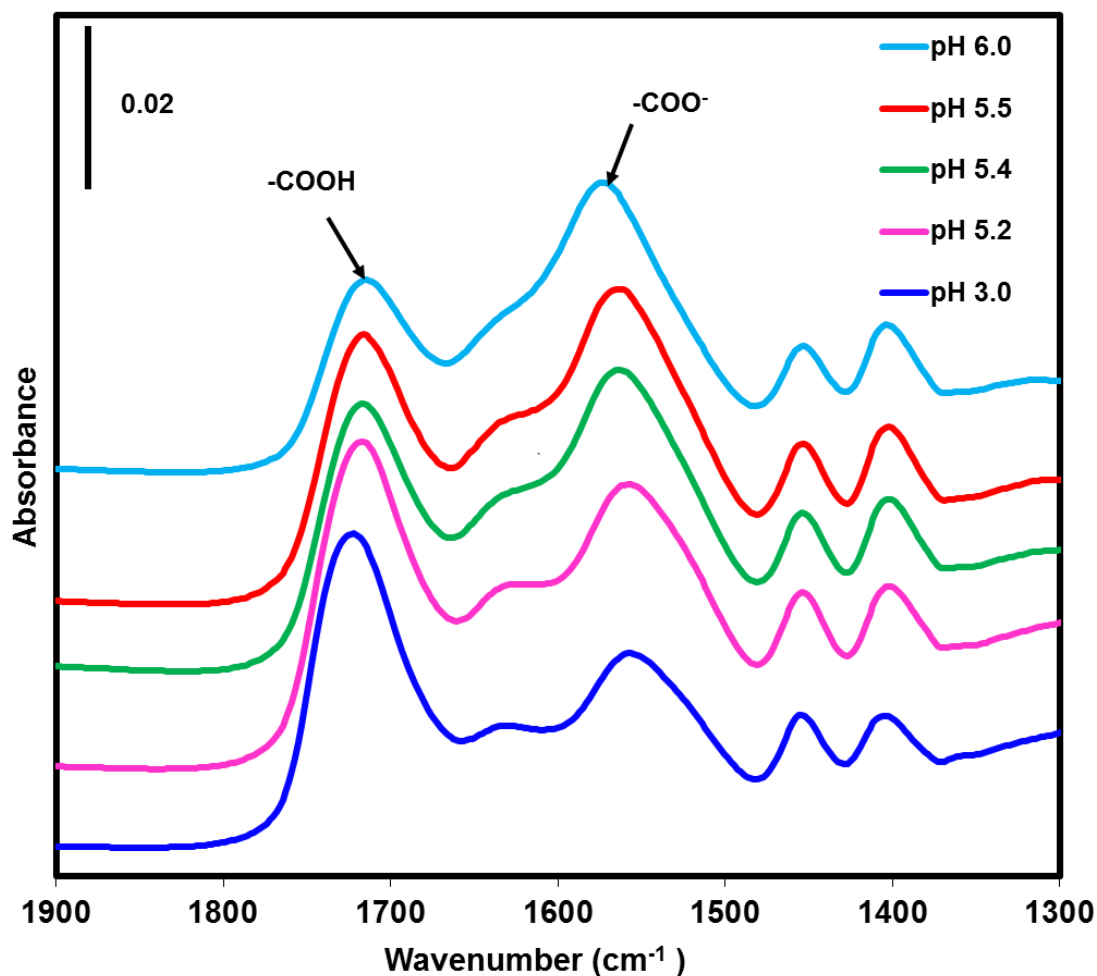


Figure 2.3. Reflectance FTIR spectra of (PAH/PAA)₅ films grown from pH 3 and later immersed in 20 mM phosphate solutions adjusted to different pH values. Prior to obtaining the IR spectra the films were rinsed only in deionized water, which based on the difference in the spectra does not alter the degree of ionization.

2.3.3. Lysozyme binding to (PAH/PAA)₅ films

We examined lysozyme sorption in (PAH/PAA)₅ films because this protein is positively charged and adsorbs through ion-exchange reactions to native (PAH/PAA)₅ coatings. These studies employed (PAH/PAA)₅ multilayers deposited from 20 mM polyelectrolyte solutions containing 0.5 M NaCl at pH 3, and lysozyme sorption occurred

from 20 mM phosphate solutions at various pH values. We used reflectance FTIR spectra (e.g. **Figure 2.4**) to determine the amide absorbance of the captured lysozyme, and prior spectra of spin-coated protein films with a variety of thicknesses allowed us to convert the amide absorbance to an equivalent thicknesses of sorbed protein (131).

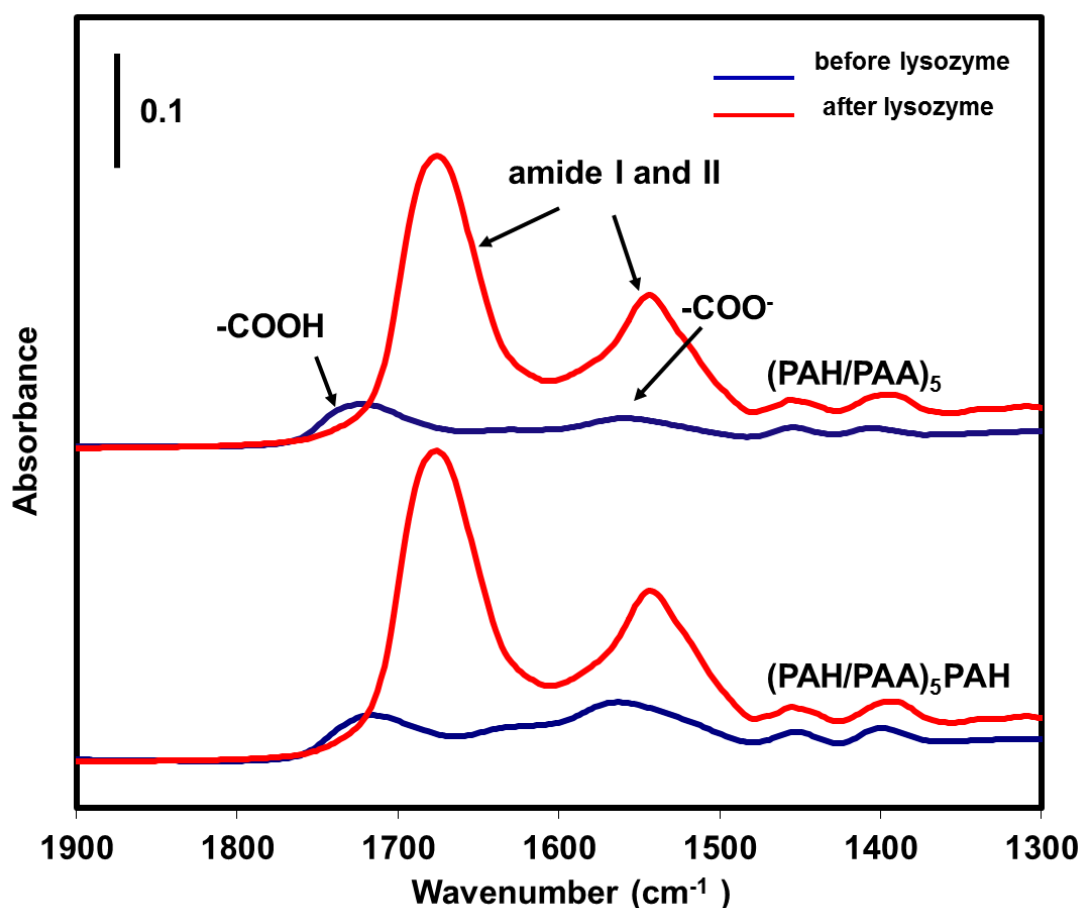


Figure 2.4. FTIR spectra of (PAH/PAA)₅ (top) and (PAH/PAA)₅PAH (bottom) films before and after sorption of lysozyme. Polyelectrolyte films were deposited from solutions containing 0.5 M NaCl at pH 3.0. Sorption of lysozyme occurred for 16 h from 20 mM phosphate buffer (pH 7.4) containing 1 mg/mL of lysozyme, and films were rinsed with water prior to obtaining the spectra.

Lysozyme sorption from pH 3 and pH 5 solutions yields lysozyme equivalent thicknesses of 7.1 ± 1.7 nm and 18.9 ± 0.2 nm, respectively. In contrast, sorption at pH

7.4 and pH 10 leads to lysozyme equivalent thicknesses around 150 nm (**Figure 2.5**). At low pH, protons effectively compete with lysozyme for -COO^- ion-exchange sites to greatly decrease binding. Notably, terminating films with PAH rather than PAA does not lead to a significant difference in lysozyme adsorption (**Figure 2.4**). Thus, the lysozyme penetrates the film during sorption. Effective lysozyme elution with 1 M KSCN is also consistent with binding by ion-exchange. Exposure to the KSCN solution for only 1 min removes 96% of the bound lysozyme. In contrast, elution with 0.1 M KSCN removes only 85% of the lysozyme in 1 h (**Table 2.1**). Binding also varies with the ionic strength of the lysozyme solution. In 20 mM phosphate buffer at pH 7.4, addition of 0.1 M NaCl to the adsorption solution decreases the effective thickness of bound lysozyme marginally from 147 ± 29 nm to 133 ± 1 nm. However, further increasing the NaCl concentration to 0.3 or 0.5 M decreases the effective lysozyme thickness to essentially zero (**Figure 2.6**). The Na^+ effectively competes with lysozyme for the ion-exchange sites.

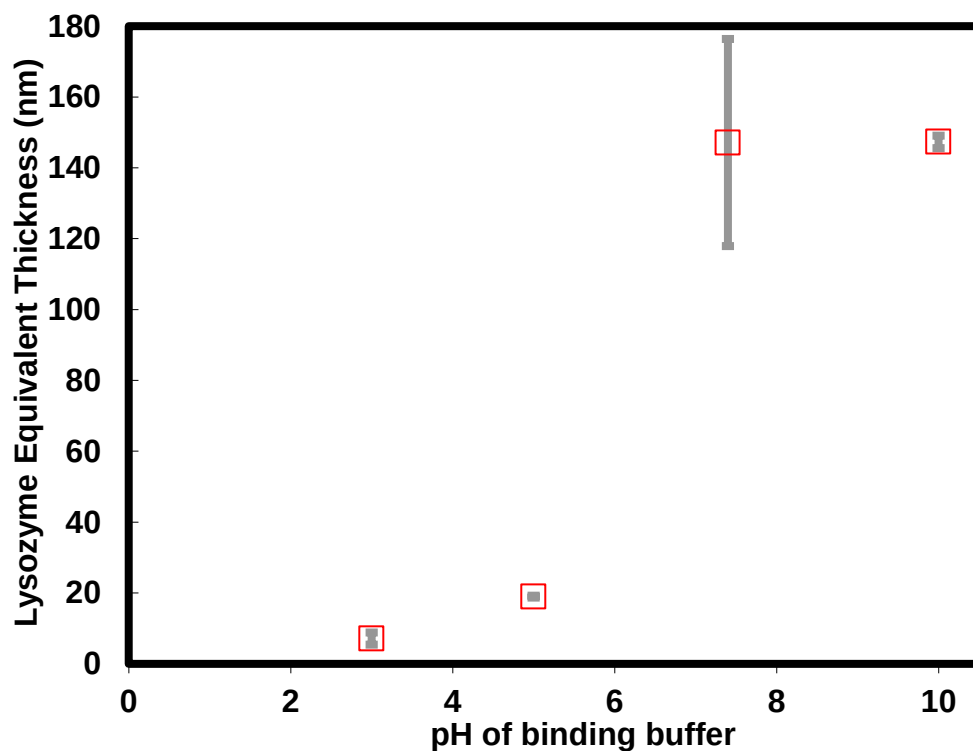


Figure 2.5. Lysozyme sorption in (PAH/PAA)₅ films as a function of the pH of the 1 mg/mL lysozyme sorption solution. (PAH/PAA)₅ coatings were deposited from solutions containing 0.5 M NaCl at pH 3. Lysozyme sorption solutions contained 20 mM phosphate adjusted to different pH values and no supporting salt. The equivalent thickness is the thickness of spin-coated lysozyme that would give an FTIR absorbance equivalent to that of the sorbed lysozyme.

Table 2.1. Efficiency of lysozyme elution from (PAH/PAA)₅ films as a function of the eluent KSCN concentration and elution times. Sorption solutions contained 1 mg/mL of lysozyme in 20 mM phosphate buffer (pH 7.4). The eluent was prepared in the same buffer. The elution efficiency was calculated from the amount of lysozyme in the film before and after elution as determined from FTIR spectra. The (PAH/PAA)₅ film was adsorbed from a pH 3 solution containing 0.5 M NaCl.

Elution time	Ratio of Eluted - to - Bound lysozyme		
	0.10 M KSCN	0.25 M KSCN	1.00 M KSCN
1 min	0.56	0.88	0.96
10 min	0.81	0.93	-
30 min	0.84	0.93	-
1 hour	0.85	0.94	-

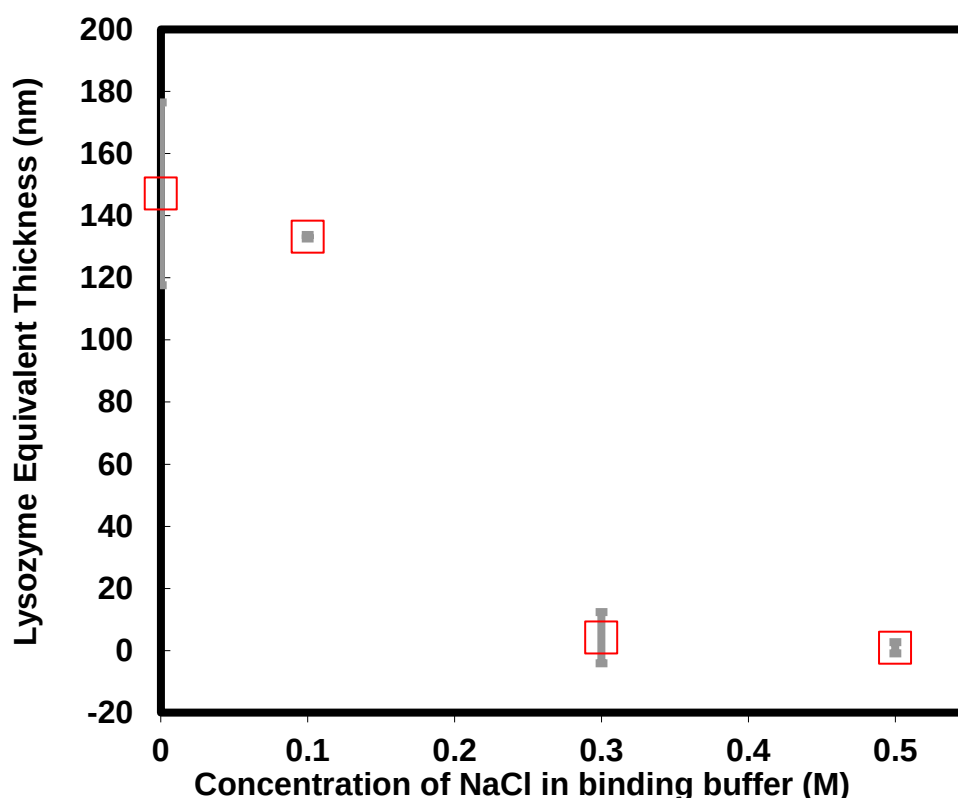


Figure 2.6. Lysozyme sorption in (PAH/PAA)₅ multilayers as a function of the concentration of NaCl in the 1 mg/mL lysozyme sorption solution. (PAH/PAA)₅ films were deposited from solutions containing 0.5 M NaCl at pH 3. The sorption solution also contained 20 mM phosphate buffer at pH 7.4. The equivalent thickness is the thickness of spin-coated lysozyme that would give a reflectance FTIR absorbance equivalent to that of the sorbed lysozyme.

The above results employed a 16-h adsorption time for lysozyme sorption to ensure equilibrium, but binding actually occurs in a few minutes. **Figure 2.7** shows how the binding capacity of a (PAH/PAA)₅ film (deposited from 0.5 M NaCl solutions at pH 3) varies with sorption time. Even after a 4-min exposure to lysozyme, the binding reaches 85% of the value at 16 h, and the difference in the amount of lysozyme sorption during 1

h and 16 h is negligible. The rapid binding suggests a highly swollen film that affords access to interior binding sites.

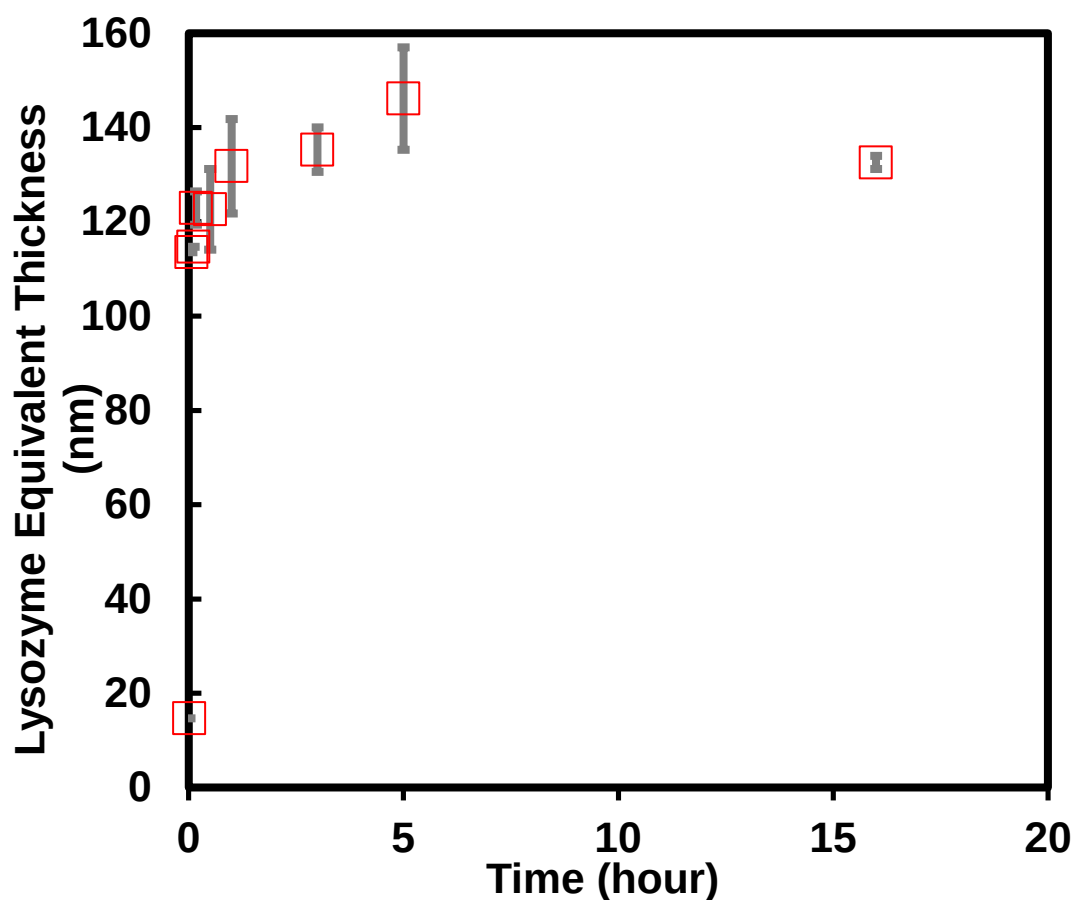


Figure 2.7. Time-evolution of lysozyme sorption in (PAH/PAA)₅ multilayers immersed in a 1 mg/mL lysozyme solution in 20 mM phosphate buffer (pH 7.4). The (PAH/PAA)₅ films were deposited from pH 3 solutions containing 0.5 M NaCl. The equivalent thickness is the thickness of spin-coated lysozyme that gives an FTIR absorbance equivalent to that of the sorbed lysozyme. The error bars represent standard deviations of measurements on 3 different films.

2.3.4. Film swelling and morphology changes upon protein sorption

To examine the extent of film swelling, we performed in situ ellipsometry with

(PAA/PAH)₅ films immersed in deionized water. The films were initially deposited at pH 3 from a 0.5 M NaCl solution. After an overnight immersion in deionized water, swelling increased the film thickness 5-fold relative to the thickness in ambient air. (Most of the swelling occurs in the first two hours of immersion). Consistent with ~80% water in the immersed coating, the film refractive index decreases from 1.50 to 1.36 after swelling. (The refractive index of water at the wavelengths of the spectroscopic ellipsometer is about 1.333.) After lysozyme binding, the dry film thickness is essentially the thickness of the water-swollen film. The coating containing bound protein swells by only 30% in water and the swollen film refractive index is around 1.50, reflective of the large amount of sorbed protein. Thus, lysozyme likely replaces water in the film.

AFM images of (PAA/PAH)₅ multilayers deposited from 0.5 M NaCl at pH 3 suggest that film morphology does not change greatly upon lysozyme sorption (**Figures 2.8 a and b**). The root-mean square roughness values for (PAA/PAH)₅ films increase from 2.0 to 3.4 nm after sorption. Given the large amount of protein binding (150 nm of lysozyme in films with an initial dry thickness of 36 nm), these images provide another evidence for diffusion of lysozyme into the interior of the film. Adsorption of multilayers of lysozyme at the surface would likely increase roughness.

Interestingly, although (PAA/PAH)₅ films deposited at pH 3 and pH 9 (from solutions containing 0.5 M NaCl) sorb similar amounts of lysozyme (around 150 nm in both cases), only the latter coating shows a large morphology change upon lysozyme sorption. With (PAA/PAH)₅ deposited at pH 9, the rms roughness increases from 2.7 to 14.7 nm upon lysozyme sorption (**Figures 2.9 a and b**). For these particular films, the

large positive charge due to both protonation of amine groups and sorption of lysozyme may cause film reconstruction (132).

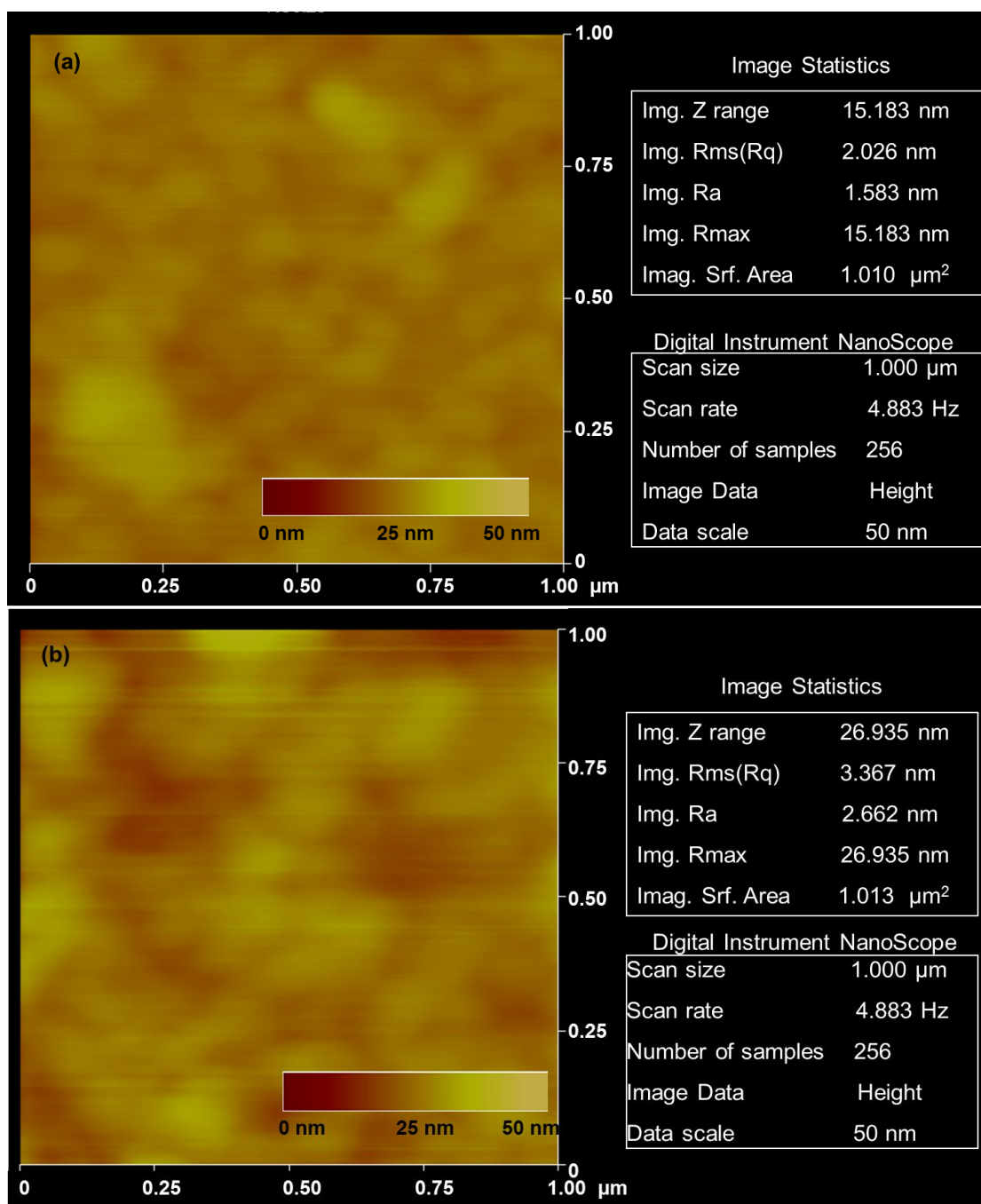


Figure 2.8. AFM images of (PAH/PAA)₅ films (a) before and (b) after sorption of lysozyme. The films were adsorbed from pH 3 solutions containing 0.5 M NaCl. Lysozyme sorption occurred from a 1 mg/mL lysozyme solution in phosphate buffer (7.4) for 16 h, and films were rinsed with deionized water prior to imaging.

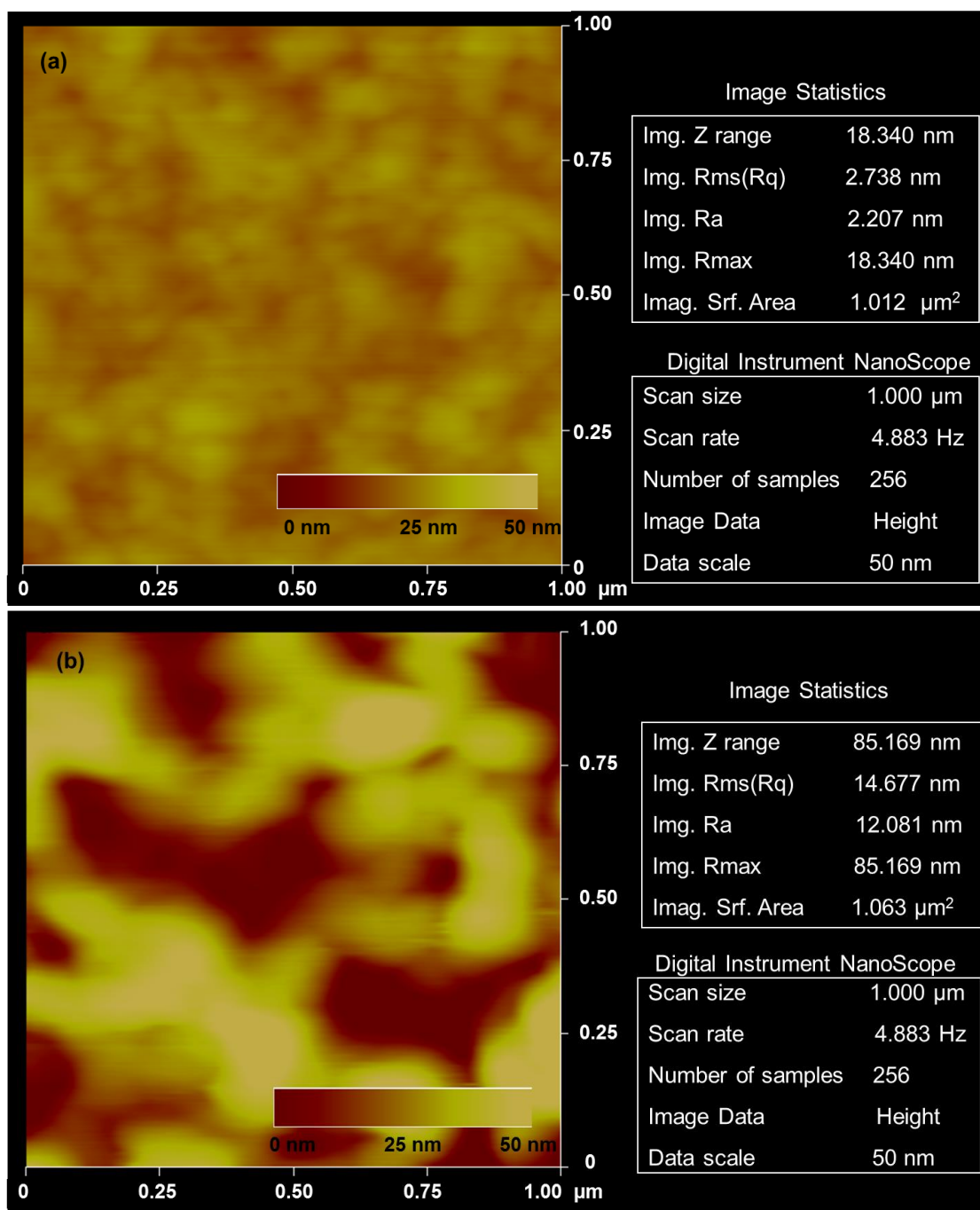


Figure 2.9. AFM images of (PAH/PAA)₅ films (a) before and (b) after adsorption of lysozyme. The films were adsorbed from pH 9 solutions containing 0.5 M NaCl. Lysozyme adsorption occurred from a 1 mg/mL lysozyme solution in phosphate buffer (7.4) for 16 h, and films were rinsed with deionized water prior to imaging.

2.3.5. Film stability and repetitive binding experiments

The high swelling of (PAA/PAH)₅ films deposited from 0.5 M NaCl at pH 3 is attractive for protein binding, but these films are not stable in neutral pH buffers in the absence of lysozyme. (They are stable in deionized water, the matrix for swelling studies.)

Figure 2.10 shows FTIR spectra of (PAA/PAH)₅ films before and after an overnight immersion in 20 mM phosphate buffers adjusted to pH 3, 5, 7.4, and 10 (no added NaCl). At pH 3, the overnight immersion does not affect the IR spectrum of the film, but after immersion in pH 7.4 or pH 10 phosphate buffer, the absorbance due to PAA decreases by about 80%. Even after immersion in pH 5 buffer, the absorbance due to PAA may decrease by 20%. However, this 20% decrease is not definitive because although the acid carbonyl absorbance near 1720 cm⁻¹ decreases, the carboxylate absorbance increases slightly. (Even though we immersed films in a pH 3 phosphate solution prior to obtaining the spectra, the degree of protonation may change slightly.). After immersion in a neutral pH 7.4 or pH 10 buffer, deprotonation of -COOH groups creates electrostatic repulsion as well as increased solubility of PAA, and this apparently leads to dissolution of much of the film. The IR spectra in **Figure 2.3** confirm partial deprotonation of -COOH groups as the ambient pH increases from 3.0 to 6.0.

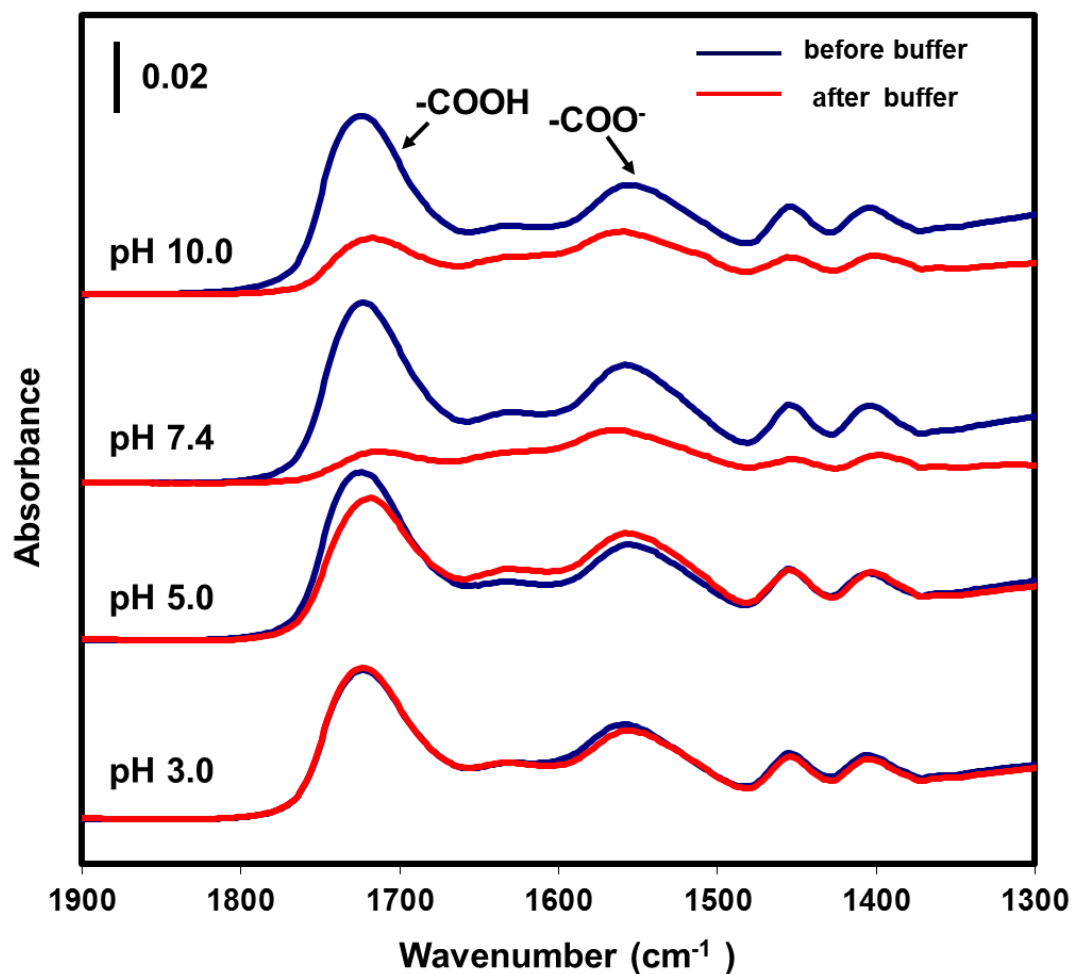


Figure 2.10. Reflectance FTIR spectra of (PAH/PAA)₅ films before and after immersion in a 20 mM phosphate solution adjusted to different pH values. (PAH/PAA)₅ films were deposited from 20 mM polyelectrolyte solutions containing 0.5 M NaCl at pH 3.0. These coatings were immersed in the buffers overnight and washed sequentially with pH 3.0 phosphate and water before obtaining FTIR spectra.

Lysozyme binding stabilizes the adsorbed PAA in (PAA/PAH)₅ films. Although binding occurs at pH 7.4 where the film is unstable, the thickness of films containing sorbed lysozyme is 180 ± 23 nm. The thickness prior to lysozyme sorption is 37 ± 6 nm. However, after elution of lysozyme with 1 M KSCN in 20 mM phosphate buffer (pH

7.4), the spectrum of the film is similar to that after immersion of the film overnight in pH 7.4 buffer, showing that the (PAA/PAH)₅ is unstable in 1 M KSCN. The protein binding could involve some replacement of PAH by lysozyme (133, 134), but given the thickness increase upon binding, there is much more sorbed lysozyme than initially adsorbed PAH.

Interestingly, after immersion in pH 7.4 phosphate buffer, the spectrum of a (PAH/PAA)₅ film is similar to that of a (PAH/PAA)₂ film (also deposited from a pH 3, 0.5 M NaCl solution). Thus, we investigated repetitive lysozyme binding to (PAA/PAH)₂ multilayers deposited at pH 3.0 and pH 9.0. **Figure 2.11** shows that 5 cycles of binding and elution on the same (PAA/PAH)₂ film deposited from a pH 3, 0.5 M NaCl solution lead to only a ~25% decrease in the binding capacity. For a (PAA/PAH)₂ film deposited from a pH 9 solution, the binding capacity decreases by 50% in the same experiment. Nevertheless, immersion of a (PAH/PAA)₂ film in pH 7.4 buffers overnight results in a 40% decrease in the carbonyl absorbance of the film (**Figure 2.12**). In contrast to films deposited from pH 3, 0.5 M NaCl solutions, (PAH/PAA)₅ films deposited at pH 9 are relatively stable at neutral pH (compare **Figure 2.10** and **Figure 2.13**), as these films contain a small fraction of –COOH groups after deposition. However, these films show a decreased protein binding capacity with repetitive use suggesting that lysozyme binding and reuse cause some instability.

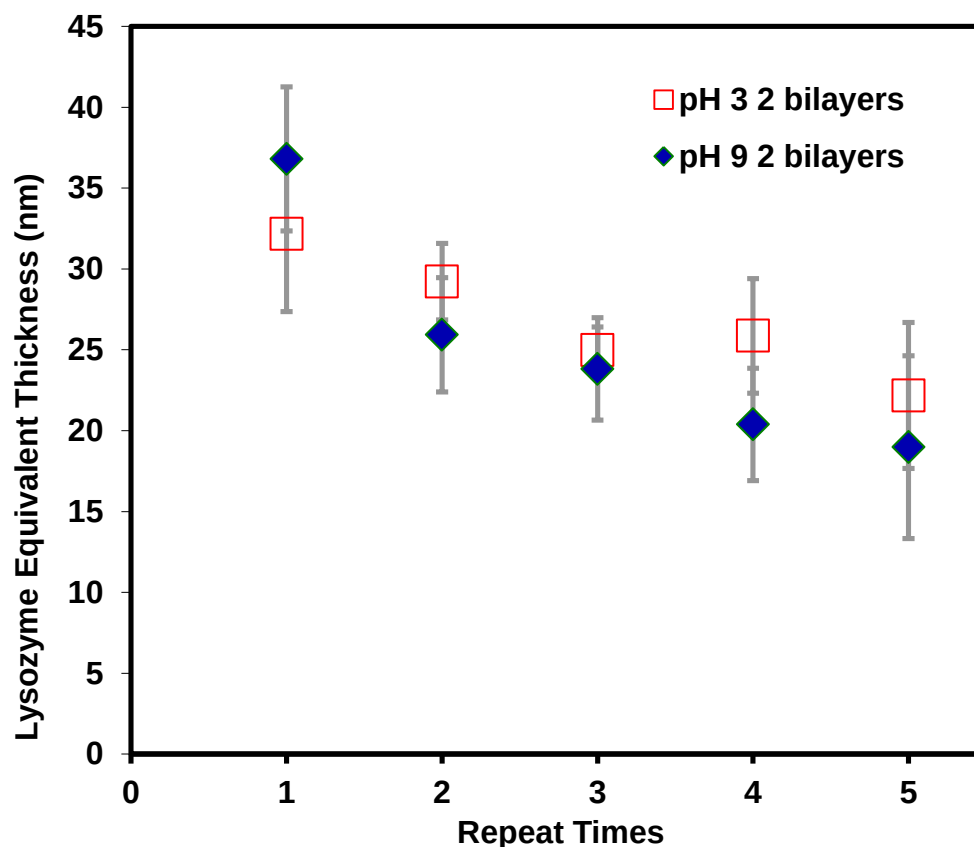


Figure 2.11. Repetitive lysozyme sorption in (PAH/PAA)₂ films from a 1 mg/mL lysozyme sorption solution. (PAH/PAA)₂ films were deposited from polyelectrolyte solutions containing 0.5 M NaCl at pH 3.0. Between each binding, the sorbed lysozyme was eluted with 20 mM phosphate buffer containing 1 M KSCN. The sorption solution also contained 20 mM phosphate buffer at pH 7.4. The equivalent thickness is the thickness of spin-coated lysozyme that would give an FTIR absorbance equivalent to that of the sorbed lysozyme.

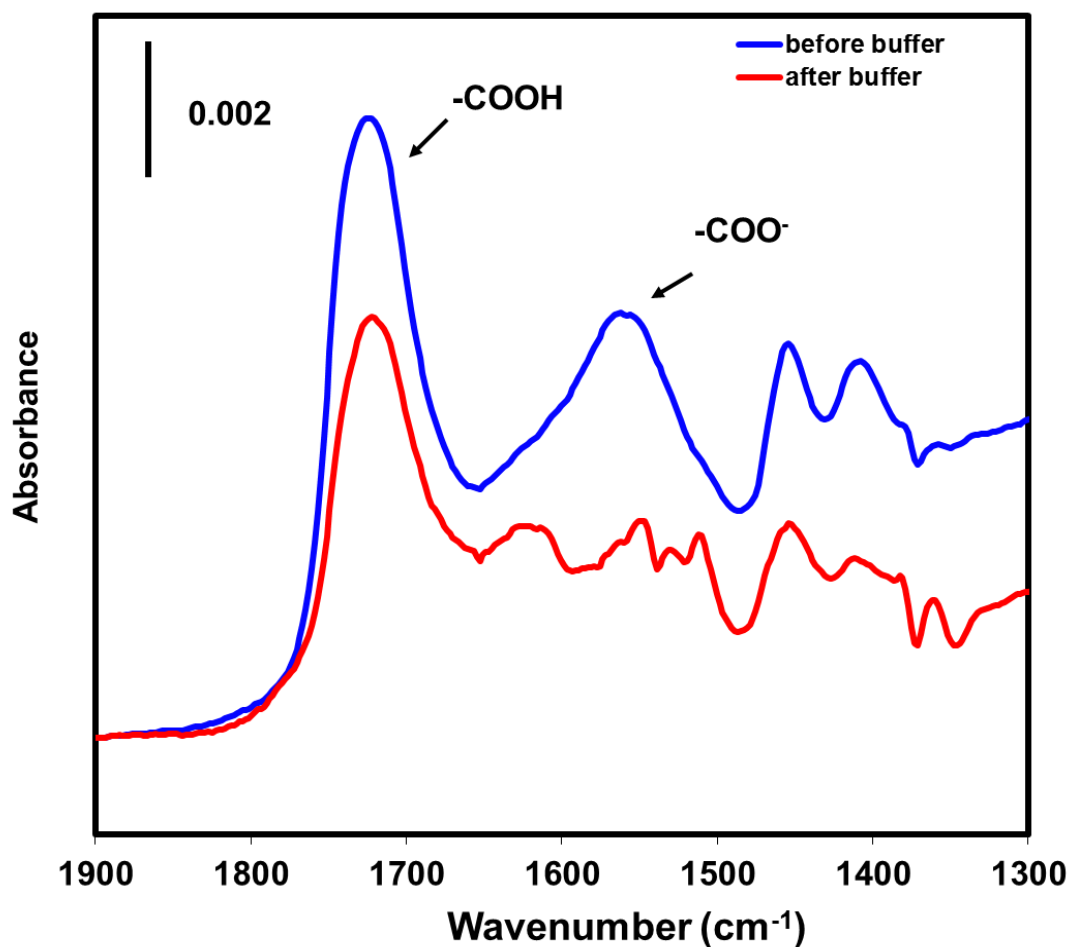


Figure 2.12. FTIR spectra of (PAH/PAA)₂ films before and after immersion in a 20 mM phosphate solution (pH 7.4). (PAH/PAA)₂ films were deposited from pH 3.0 polyelectrolyte solutions containing 0.5 M NaCl. The film was immersed in the buffer overnight and washed with pH 3.0 phosphate buffer and water before FT-IR measurements.

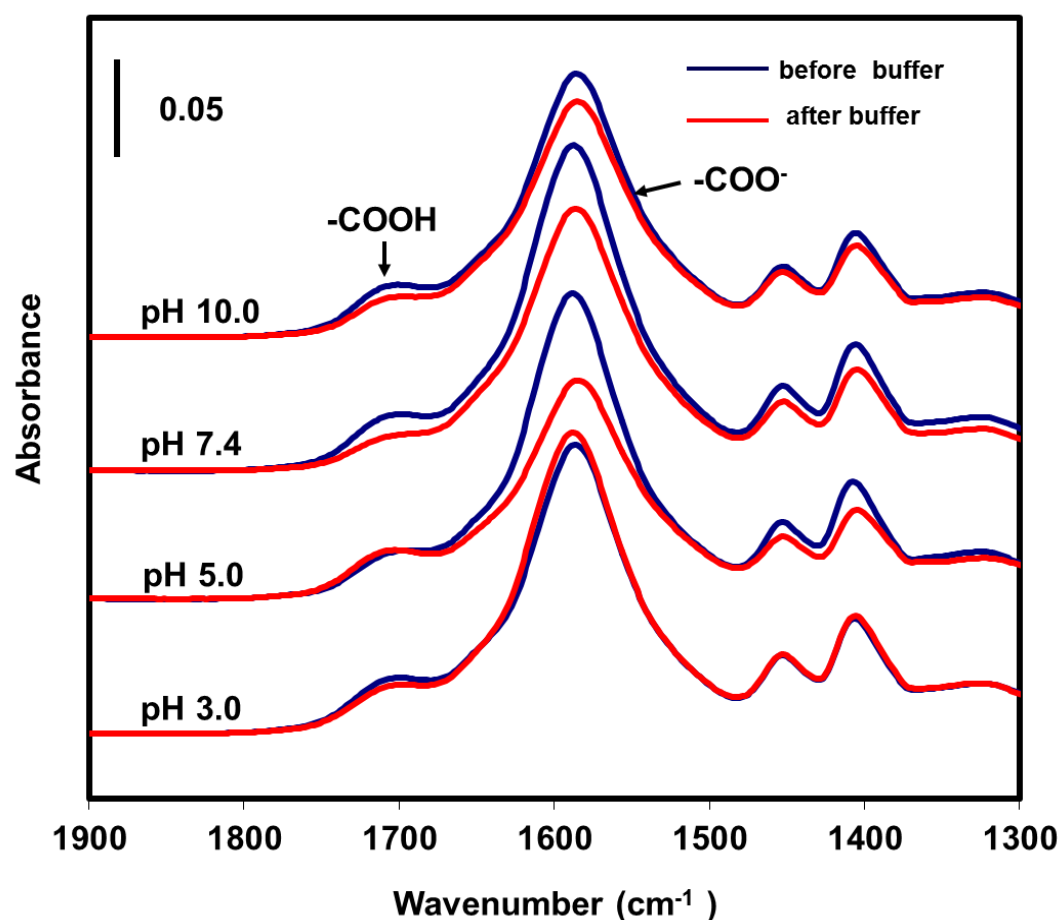


Figure 2.13. FTIR spectra of (PAH/PAA)₅ films before and after immersion in a 20 mM phosphate solution adjusted to different pH values. (PAH/PAA)₅ films were deposited from pH 9.0 polyelectrolyte solutions containing 0.5 M NaCl. The films were immersed in the buffers overnight and washed with pH 9.0 buffer and water before FT-IR measurements.

2.4. Conclusions

LbL adsorption of PAA and PAH gives PEMs whose properties depend on the deposition pH. Films grown from pH 3 swell by 500% in pH 7.4 buffer and capture multilayers of lysozyme throughout the film structure. Lysozyme binding depends on the

pH and ionic strength of the binding buffers because of the ion-exchange mechanism. At neutral pH and above, deprotonation of the -COOH groups in the film increases swelling and creates ion-exchange sites for lysozyme binding. In contrast, electrolytes in the elution solution can compete with the protein for ion-exchange sites to enable efficient protein elution from the films. Films adsorbed on Au-coated wafers at pH 3 bind large amounts of protein, but they are not stable at neutral pH in the absence of protein. However, film stability may be greater on other substrates such as nylon membranes where hydrophobic interactions between polyelectrolytes and initial layers occur.

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Part of this chapter is adapted from our published paper in *Langmuir* with permission of the American Chemical Society (Bhattacharjee, S., Dong, J., Ma, Y., Hovde, S., Geiger, J., Baker, G., and Bruening, M. L. *Langmuir*, **2012**, 28, 6885-6892.). Professor Jeffery McCutcheon at the University of Connecticut provided the electrospun nylon membranes, and Salinda Wijeratne prepared the newly designed polyelectrolyte (manuscript is in preparation).

Chapter 3. Purification of His-tagged protein using functionalized membranes

Chapter 2 examined protein adsorption in model films on flat substrates. This chapter describes the adsorption of poly(acrylic acid) (PAA)/polyethyleneimine (PEI)/PAA films in porous membranes and subsequent derivatization of these films to capture His-tagged protein. His-tagged proteins bind to Ni^{2+} -nitrilotriacetate (NTA) complexes appended to the PAA/PEI/PAA films. (Note that a previous study showed slightly higher lysozyme binding when polyelectrolyte films contain PEI rather than PAH (1), so this work employs PEI as a polycation.) We investigate both metal-ion binding and protein binding and compare the recovery and specificity of protein capture to purification with commercial resins. To simplify the membrane modification process and decrease cost, Salinda Wijeratne of our group developed a new polymer that I use to modify the membranes for His-tagged protein purification.

3.1. Introduction

Since the approval of recombinant insulin as a therapeutic agent in 1982 (2), the global therapeutic protein market has expanded rapidly with the continuous growth of biotechnologies (3, 4). However, purification of proteins remains a bottleneck in their production because of the complexity of protein mixtures. Affinity adsorption of target proteins to ligands immobilized in packed columns is probably the most powerful step in protein purification (5, 6), and this method is also vital to obtaining purified protein for fundamental research.

To facilitate isolation of target proteins, affinity tags (usually peptides or fusion proteins) are attached to these proteins through gene engineering (7). Such tags include polyhistidine (His) (8), the octapeptide FLAG (9), glutathione S-transferase (10) and maltose binding protein (11). Among these tags, His-tag is the most common because it often does not alter protein properties, and purification is relatively inexpensive (12). The His-tag, usually 6 histidine residues, chelates to immobilized metal ions complexed on resins (8). However, resin-based protein purification requires relatively long separation times due to the slow diffusion of macromolecules to binding sites in nanopores, and slow purification is especially troublesome for unstable proteins (5, 13-15).

As summarized in Chapter 1, porous membranes modified with affinity ligands overcome some of the limitations of resin-based affinity techniques. For His-tagged protein purification, the Bruening group successfully developed affinity membranes through growth of polymer brushes in membrane pores (16-21). This chapter aims to create His-tagged protein-purification membranes using layer-by-layer adsorption of

PEMs, which is much easier than surface-initiated polymerization. Based on our previous studies, membranes containing PAA/PEI/PAA films deposited at pH 3 provide high lysozyme capture even when proteins spend only 35 ms in membranes, and the membranes are stable for at least 6 repetitive purifications (1, 22). Thus, below I examine metal-ion binding and His-tagged protein purification using Ni^{2+} - or Cu^{2+} -NTA-functionalized PAA/PEI/PAA films. The work also examines adsorption of PAA/PEI/PAA-NTA- Cu^{2+} on mats of electrospun nylon fibers. Finally I investigate protein binding to PEMs containing poly(2,2-(5-acrylamido-1-carboxypentylazanediyl) diacetic acid) (PNTA), a new polymer synthesized by Salinda Wijeratne, to simplify the membrane fabrication.

3.2. Experimental section

3.2.1. Materials

Hydroxylated nylon (LoProdyne® LP, Pall, 1.2 μm pore size, 110 μm thick) and electrospun nylon fibers membranes (obtained from Professor Jeffery McCutcheon at the University of Connecticut) were cut into 25 mm-diameter discs prior to use. Unless specified, all proteins and chemicals were obtained from Sigma-Aldrich. Coomassie protein assay reagent (Thermo Scientific), Histidine6-tagged Ubiquitin (HisU, human recombinant, Enzo Life Sciences), Concanavalin A from *Canavalia ensiformis* (Con A, Jack bean), albumin from chicken egg white (Ovalbumin); lysozyme from chicken egg white; bovine serum albumin (BSA), and β -Lactoglobulin B from Bovine milk were used as received. His-tagged COP9 signalosome complex subunit 8 (CSN 8) was

overexpressed in BL21DE3 cells by Stacy Hovde in Professor James Geiger's group. Buffers were prepared using analytical grade chemicals and deionized water (Milli-Q, 18.2 MΩ cm). Polyethyleneimine (PEI, branched, Mw = 25,000), poly(sodium 4-styrenesulfonate) (PSS, Mw = 70 000), poly(acrylic acid) (PAA, Mw = 90,000, 25% aqueous solution, Polysciences), TWEEN-20 surfactant, *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), and *N*_α*N*_α-Bis(carboxymethyl)-L-lysine Hydrate (Aminobutyl NTA) were used without further purification. PNTA was synthesized by Salinda Wijeratne and will be described in his dissertation.

3.2.2. Membrane modification

Membrane discs were cleaned for 10 min with UV/ozone and placed in a homemade Teflon holder (similar to an Amicon cell) that exposed 3.1 cm² of external membrane surface area. Subsequently, a 5-mL solution containing PAA (0.02 M with 0.5 M NaCl) was circulated through the membrane for 20 min at a flow rate of 1 mL/min using a peristaltic pump (**Figure 3.1**). Following a literature protocol, additional polycation (PEI, 2 mg/mL without NaCl in the deposition solution) and polyanion (PAA, with 0.5 M NaCl in the deposition solution) layers were deposited similarly. After adsorption of each polyelectrolyte, 20 mL of water was passed through the membrane at the same flow rate. The pH values of PAA, PAH, and PEI deposition solutions were adjusted to 3 with 1 M NaOH or 1M HCl for PAA/PEI/PAA films. For the PNTA-containing PEMs, the concentration of polyelectrolytes in deposition solutions was 1 mg/mL. To optimize

metal-ion binding, we investigated adsorption with and without 0.5 M NaCl in the polyelectrolyte solution. We also employed PNTA, PSS and PAA as the first layer attached to the membrane to initiate the film growth. In some cases, to grow thicker films in the membrane, the pH of polyanion solutions, including PNTA, PSS and PAA, was set to 3, whereas the PAH solution was adjusted to pH 3 or pH 9. The adsorption and rinsing process was the same as for PAA/PEI/PAA films. Membrane hydraulic permeabilities were determined as described previously by measuring the mass flux through the membrane at fixed pressures (16).

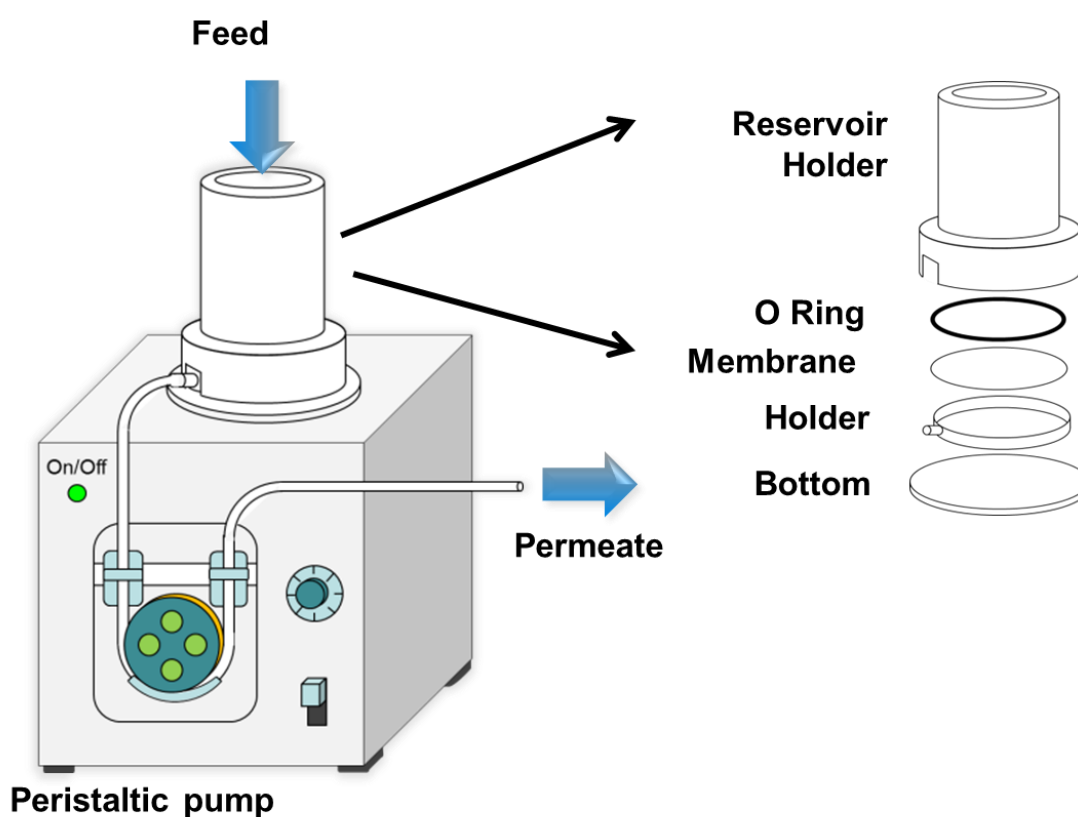


Figure 3.1. Apparatus for membrane modification and protein-binding studies.

Drawn by Zhefei Yang.

To derivatize PAA side chains in adsorbed films, 10 mL of 0.1 M NHS, 0.1 M EDC in water was circulated through the membrane for 1 h prior to rinsing with 20 mL of

deionized water and 20 mL of ethanol. Subsequently, 10 mL of aqueous aminobutyl NTA (0.1 M, adjusted to pH 10.2 with NaOH) was circulated through the NHS-modified substrate for 1 h followed by rinsing with 20 mL of water. Finally, the NTA-Cu²⁺ (or Ni²⁺) complex was formed by circulating 10 mL of aqueous 0.1 M CuSO₄ (or NiSO₄) through the membrane for 1 h followed by rinsing with 20 mL of water. The substrate was dried with N₂ prior to protein binding.

3.2.3 Metal-ion binding and leaching

After derivatization of PEMs with Ni²⁺ or Cu²⁺, bound metal ions were eluted with two 5-mL aliquots of 0.1 M Ethylenediaminetetraacetic acid (EDTA) (pH 7.6). The concentrations of metal ions were determined with atomic absorption spectroscopy (AAS) using calibration curves with metal-ion concentrations ranging from 0 ppm to 10 ppm in 0.1 M EDTA. The metal-ion binding capacity was calculated by dividing the mass of eluted metal ions by the volume of membrane, which is about 0.035 cm³ (the membranes are 110 μm thick and have an exposed area of 3.1 cm²). To study the metal-ion leaching, the membrane was washed sequentially with 5 mL of four different buffers and 0.1 M EDTA (pH 7.4). The buffer compositions were: binding buffer- 20 mM phosphate buffer with 0.3 M NaCl and 10 mM imidazole; washing buffer I- 20 mM phosphate buffer with 0.15 M NaCl and 0.1% Tween-20; washing buffer II- 20 mM phosphate buffer with 0.15 M NaCl and 45 mM imidazole; and elution buffer- 20 mM phosphate buffer with 0.5 M NaCl and 0.3 M imidazole. The pH of all buffers was 7.4. The samples were diluted 5-fold with 0.1 M EDTA before the metal-ion concentration was determined with AAS.

3.2.4 Protein binding

A solution of lysozyme (0.3 mg/mL) in 20 mM phosphate buffer (pH 7.4) was pumped through the modified membrane at a flow rate of 1 mL/min, and the permeate was collected for analysis at specific time intervals. Subsequently, the membrane was rinsed with 20 mL of washing buffer I 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. Unless specified, flow rates were 1 mL/min.

Con A solutions (0.3 mg/mL) were prepared in 20 mM phosphate buffer at pH 6 because this protein is not stable at pH 7.4. For Con A, washing buffer I and elution buffer were also adjusted to pH 6. The membranes were loaded with buffered protein solution, rinsed with 20 mL of washing buffer I followed by 20 mL of phosphate buffer at pH 6, and eluted with 8-9 mL of imidazole elution buffer (see above). The concentrations of protein in loading, rinsing, and eluate solutions were determined using a Bradford assay (16).

3.2.5. Purification of HisU from a model protein mixture

To test protein binding specificity and recovery, we prepared a solution containing 0.05 mg/mL (each) of His-U, Con A, BSA, Ovalbumin, and β -Lactoglobulin B in 20 mM phosphate buffer (pH 7.4). Ten mL of this protein solution was passed through a PAA/PEI/PAA-NTA-Ni²⁺-modified membrane at 1.5 mL/min after the membrane was equilibrated with 20 mL of phosphate buffer. Subsequently, the membrane was washed with 20 mL of washing buffer I and 20 mL of phosphate buffer. The bound protein was

initially eluted with 10 mL (2 mL for each aliquot) of elution buffer to study the elution efficiency, but the first aliquot contained more than 95% of the bound protein. The purity of eluted protein was examined by Polyacrylamide gel electrophoresis (SDS-PAGE, 4-20% gradient gel from Bio-Rad with standard Coomassie blue staining protocols).

3.2.6. Protein isolation from a cell extract

At a flow rate of 1.5 mL/min, 3 mL of lysate supernatant diluted 1:2 using binding buffer was passed through a PAA/PEI/PAA-NTA-Ni²⁺-modified membrane that was previously equilibrated with lysate buffer (20 mM pH 8 phosphate buffer containing 10 mM imidazole and 300 mM NaCl). To be consistent with the lysis buffer, all the binding, washing and elution buffers were adjusted to pH 8.0. After washing with 20 mL of washing buffer I, 20 mL of washing buffer II and 20 mL of phosphate buffer at a flow rate of 5 mL/min, protein elution and gel electrophoresis followed the same procedure for HisU purification.

To compare the membrane to commercial products, CSN 8 was also purified with a spin column packed with HisPur Ni-NTA resins from Thermo Scientific (0.5 mL resins in each 1.5 mL column). Before use, the column was equilibrated with 1 mL of binding buffer for 10 min and centrifuged for 2 min to remove the binding buffer. Subsequently, 3 mL of 3-fold diluted cell extract was loaded on the column in two steps (1.5 mL for each loading). In each loading step, the column was incubated for 10 min before centrifuging for 2 min. The column was then sequentially washed with 2 aliquots of washing buffer I, washing buffer II and binding buffer before the protein was eluted with 2 aliquots of

elution buffer. As with the loading, 10 min of incubation and 2 min of centrifugation were applied for each washing and elution step. The whole process took about 3 hours including the protein loading, washing and elution.

3.3. Results and discussion

Membranes are attractive for rapid protein purification, but they often have a low binding capacity. Prior work showed that adsorption of PEMs in membrane pores greatly increases lysozyme-binding capacities (1). However, the PEM must not plug membrane pores. The following four subsections discuss the permeability of PAA/PEI/PAA-modified membranes and derivatization of these membranes for capture of His-tagged protein from cell extracts. Subsequent subsections examine modification of electrospun nylon fibers as well as adsorption of a NTA-containing polymer to simplify membrane modification.

3.3.1. Permeability of membranes with PAA/PEI/PAA films

Chapter 2 showed that (PAH/PAA)_n films can serve as coatings with high lysozyme-binding capacities. Bhattacharjee and coworkers used PAA/PAH/PAA and PAA/PEI/PAA films to modify hydroxylated nylon membranes and found that the lysozyme binding capacity was higher with PEI as the polycation (1). As with films on flat surfaces, adsorption pH plays an important role in the properties of PAA/PEI/PAA films (23). With an adsorption pH of 3, membranes modified with PAA/PEI/PAA captured over 100 mg of lysozyme per cm³ of membrane, and the lysozyme breakthrough curve showed little dependence on flow rate (1). Based on those

promising results, much of the research in this chapter examines membranes modified with PAA/PEI/PAA films deposited at pH 3.

Plugging of pores is always a potential problem when modifying membranes by adsorption. Moreover, directly quantifying the amount of thin film adsorption in highly porous substrates is difficult. Nevertheless, permeabilities give an indication of changes in membrane pore size distribution due to modification (**Table 3.1**). The permeability of membranes to pH 7.4 phosphate buffer (20 mM) decreased from 78 to 20 mL/(cm² min atm) when comparing a bare membrane and a membrane containing a PAA/PEI/PAA film. Although this is a significant decline in permeability, rapid flow through the membrane can still occur using a simple peristaltic pump. In contrast, the permeability of PAA/PEI/PAA-modified membranes to deionized water after treatment with buffer is <1 mL/(cm² min atm). This suggests that at low ionic strength (deionized water), unscreened electrostatic repulsions lead to high film swelling. Rinsing membranes with 2.7 mM HCl protonates –COOH groups, and the presumed resulting collapse of polymers restores water permeability to 96 mL/(cm² min atm). The lower permeability in pH 7.4 buffer than in deionized water after HCl treatment is consistent with deprotonation of the polymer at neutral pH, and these results are similar to prior studies of pH-responsive membranes (24-26).

We roughly estimated the pore size under each condition using the Hagen-Poiseuille equation, which states that the volumetric flux is proportional to d^4 , where d is the diameter of the open fraction of the pore (27). Assuming that the bare membrane has a pore size of 1.2 μm , the pore diameter decreased to $\sim 1 \mu\text{m}$ after PAA/PEI/PAA

adsorption and then declined to 0.21 μm after exposure to buffer and then water. As discussed above, contact with 2.7 mM HCl opens the pore, presumably due to minimal film swelling in the protonated state. Water flux also dropped (relative to the membrane treated with HCl) after exposure to 1 M KSCN in phosphate buffer. This demonstrates that the film is stable in high salt concentrations. A subsequent exposure to 2.7 mM HCl again restores flux.

Table 3.1. Water or buffer permeabilities of bare and PAA/PEI/PAA-modified nylon membranes in sequential measurements. Buffer: 20 mM phosphate buffer (pH 7.4); KSCN: 1M KSCN in 20 mM phosphate buffer (pH 7.4) and HCl: 2.7 mM HCl. The pressure was maintained at 0.68 atm and the unit for permeability is mL/(cm² min atm). The drop in permeability is calculated with respect to the water permeability of the bare membrane.

	Bare membrane		PAA/PEI/PAA modified membrane					
	Water	Buffer	water	Buffer	Water after buffer rinse	Water after HCl rinse	Water after KSCN rinse	Water after HCl rinse
average	115	77.8	58.9	20.0	0.1	95.8	0.1	87.6
drop (%)	0.0	27.7	48.6	82.5	99.9	16.4	99.9	23.5
pore size/ μm	1.20	1.09	1.02	0.78	0.21	1.15	0.23	1.12

^aThe table shows data for one bare and one modified membrane. Corresponding experiments on other membranes showed less than 20% deviation from these values.

3.3.2. Functionalization of membranes with NTA and studies of metal-ion binding

During purification, His-tags chelate metal ions in complexes immobilized on a solid

support (8). Although PAA contains -COOH groups, isolated carboxylates do not bind metal ions strongly. Thus, to effectively create immobilized metal-ion complexes, we anchored aminobutyl NTA to PAA/PEI/PAA films using the strategy in **Figure 3.2**. Films deposited at pH 3 contain a high density of free (not paired with ammonium groups) -COOH groups that are available for reaction with aminobutyl NTA through NHS/EDC coupling (1, 16, 18). The immobilized NTA strongly complexes ions such as Cu^{2+} or Ni^{2+} .

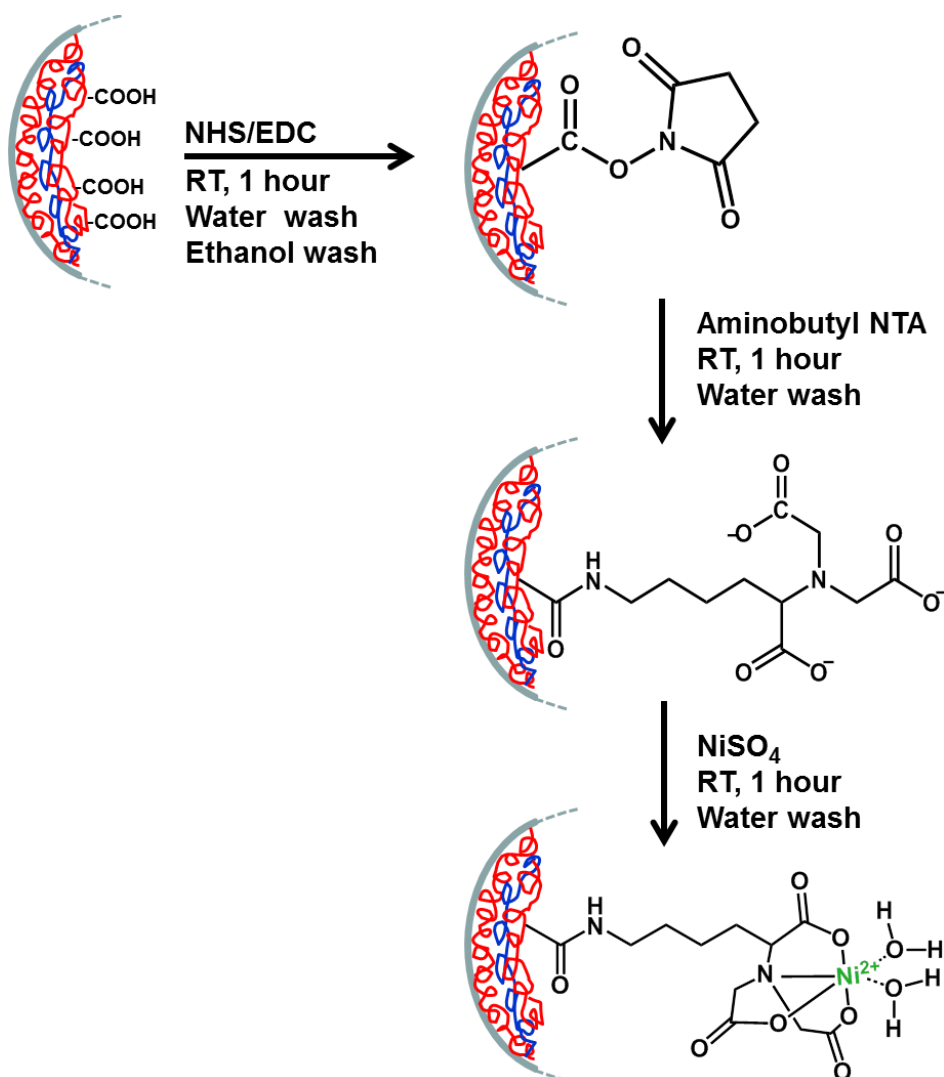


Figure 3.2. Schematic illustration of PAA/PEI/PAA derivatization using NTA- Ni^{2+} .

RT: room temperature. Reproduced from reference 1 with permission of the American Chemical Society.

PAA/PEI/PAA-NTA-modified membranes have binding capacities of 18 mg/cm^3 and 9.5 mg/cm^3 for Cu^{2+} and Ni^{2+} , respectively. However, in addition to the formation of NTA-metal ion complexes, weak Cu^{2+} and Ni^{2+} binding to underivatized -COOH groups may complicate the interpretation of metal-ion binding data. Thus, we compared Cu^{2+} or Ni^{2+} binding to PAA/PEI/PAA and PAA/PEI/PAA-NTA membranes. For both Cu^{2+} and Ni^{2+} , the binding capacities for the NTA-derivatized membranes were $\sim 30\%$ higher than

those for underivatized membranes, but significant binding occurs to the PAA/PEI/PAA film. Derivatization will likely decrease binding to free COO^- groups, but it still occurs. To distinguish between metal-ion binding to COO^- and NTA groups, I tried to selectively elute the metal ions from the membranes using buffers that contain different concentrations of imidazole. **Table 3.2** shows the percentage of Cu^{2+} or Ni^{2+} (out of the total immobilized Cu^{2+} or Ni^{2+}) leached in different solutions. In the case of Cu^{2+} , about 10% more Cu^{2+} was eluted from membrane without NTA by the 10 mM imidazole in binding buffer but the opposite trend occurred in the 45 mM imidazole in washing buffer II. As a result, the percentage of Cu^{2+} eluted in EDTA was almost the same for both membranes. For Ni^{2+} , the high percentage of ions leached by the binding buffer from the PAA/PEI/PAA-NTA membrane clearly demonstrates that Ni^{2+} also binds to COOH groups. We tried unsuccessfully to selectively elute Ni^{2+} bound to underivatized COOH groups, but the percentages of leached Ni^{2+} ions were similar for PAA/PEI/PAA-NTA and PAA/PEI/PAA modified membranes. Low metal-ion leaching is important to avoid contaminating protein solutions, so we designed a new polyelectrolyte, PNTA, discussed in section 3.3.6

Table 3.2. Cu^{2+} or Ni^{2+} leaching from membranes modified with PAA/PEI/PAA-NTA and PAA/PEI/PAA. The numbers in the table represent the percentage of metal-ion loss (relative to the total loaded metal) in each solution.

Five mL of each solution was passed through the membrane during the leaching.

Membranes	Metal ions	Binding buffer	Washing buffer I	Washing buffer II	Elution buffer	0.1 M EDTA
PAA/PEI/PAA-NTA (%)	Cu^{2+}	17.1	6.8	26.6	31.8	17.7
	Ni^{2+}	55.9	2.3	9.6	8.9	23.2
PAA/PEI/PAA (%)	Cu^{2+}	26.8	5.5	14.9	35.0	13.6
	Ni^{2+}	56.4	3.4	10.1	14.1	16.0

3.3.3. Purification of HisU from model protein mixtures

To demonstrate the selectivity of PAA/PEI/PAA- Ni^{2+} -modified membranes for His-tagged protein capture, we first separated HisU from a mixture of HisU, Con A, BSA, Ovalbumin, and β -Lactoglobulin B. These model proteins, except HisU, do not have His-tags and serve as contaminating proteins in this experiment. SDS-PAGE analysis of the mixed-protein solution exiting the membrane suggests successful removal of HisU (**Figure 3.3 lane 3**), whereas the eluate shows only bands due to HisU (**Figure 3.3 lane 4**, note that even the as-received HisU shows two bands). Thus, the membranes are highly selective for capturing HisU. Five aliquots of 2 mL elution were used to elute the bound protein, but the elution mainly occurred during the first 2 mL and no bands appeared for the second eluate as shown in **Figure 3.3 lane 4 and 5**.

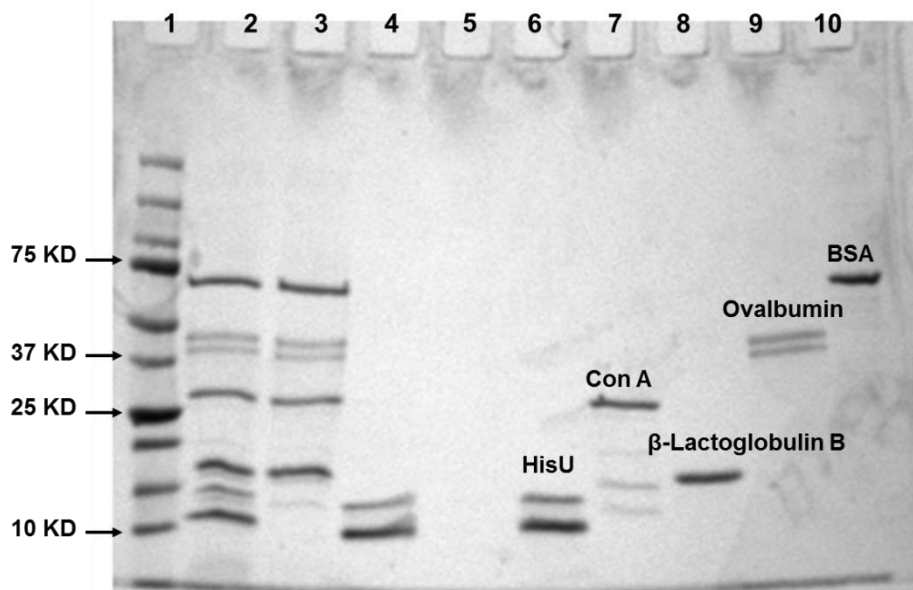


Figure 3.3. SDS-PAGE analysis (Coomassie blue staining) of HisU purification from a mixture of BSA, Ovalbumin, Con A, β -Lactoglobulin B, and HisU: lane 1- a protein ladder; lane 2- the protein solution; lane 3- the protein solution after passage through a PAA/PEI/PAA-NTA- Ni^{2+} -modified membrane; Lane 4- the 1st eluate from the membrane, lane 5 –the 2nd eluate from the membrane; Lanes 6 to lane 10 are the pure model proteins. Two μg of each protein was loaded in each lane. The eluate should contain 2 μg of HisU if all the protein was captured and eluted.

The purity of the eluted HisU allowed us to use Bradford assays to quantify the recovery from the membrane purification. (These assays examine changes in Coomassie blue absorbance due to interactions with the protein.) Based on the absorbance of a series of standard solutions of HisU in elution buffer and the absorbance of the eluate solution, the recovery was $95 \pm 3\%$. Thus, less than 10% of the protein was lost in the purification process.

3.3.4. Purification of His-tagged CSN 8 from cell extracts

To further demonstrate the high selectivity and potential biological applications of PAA/PEI/PAA-NTA-Ni²⁺-modified membranes, we purified His-tagged CSN 8 protein from a whole-cell extract. **Figure 3.4** shows the SDS-PAGE analysis of the cell extract (**lane 2**) and purification of His-tagged CSN8 using a spin column packed with Ni²⁺-resin or a PAA/PEI/PAA-NTA-Ni²⁺-modified membrane. Remarkably, the membrane eluate contains only one strong band with a purity of above 95% based on the darkness of the band. The elution (**lanes 7 and 8**) from the resin-containing spin column is less efficient. The first eluate from the spin column shows clear bands for contaminating proteins, and the elution was not complete. Additionally, compared to the commercial Ni²⁺ spin columns, the membrane purification process takes less time. A complete membrane purification requires less than 20 min, including passing cell lysate through the membrane, 3 washes using different buffers and elution, while for the spin column, the whole process takes about 3 hours. This membrane is in the final stage of commercialization, and we expect products to appear in 2015. With the commercial membrane holder, the whole process only requires 5 min.

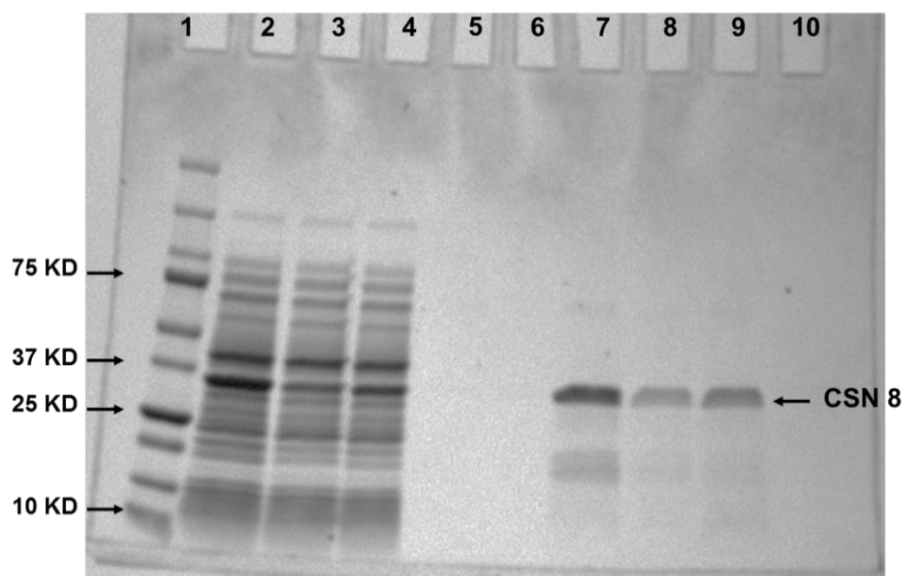


Figure 3.4. SDS-PAGE analysis (Coomassie blue staining) of His-tagged CSN8 purified from a cell extract: lane 1- a protein ladder; lane 2- a cell extract from BL21DE3 cells with overexpressed His-tagged CSN8 protein; lane 3- the cell extract after passing through a spin column packed with Ni^{2+} -resins; lane 4- the cell extract after passing through a PAA/PEI/PAA-NTA- Ni^{2+} -modified membrane; Lane 5-column wash; Lane 6-membrane wash; Lane 7- the 1st eluate from the spin column; lane 8- the 2nd eluate from spin column, lane 9- the 1st eluate from the membrane and lane 10-the 2nd eluate from the membrane.

3.3.5. Modification of electrospun fibers

Mats of electrospun nylon fibers have a high porosity and could potentially serve as membrane supports with high permeability and protein-binding capacities. Thus, we experimented with layer-by-layer modification of nylon fibers electrospun on a poly(ethylene terephthalate) (PET) support. Provided by professor Jeffery McCutcheon, the mats contain 30 μm of spun fiber on a 30 μm -thick PET support. The modification procedure was the same as that for the nylon membranes described above. Lysozyme

binding to PAA/PEI/PAA-modified fibers and Con A binding to PAA/PEI/PAA-NTA-Cu²⁺-modified fibers served as metrics for the performance of the modified fibrous mats.

3.3.5.1. Lysozyme binding

PAA/PEI/PAA multilayers deposited at pH 3 on the electrospun nylon membrane contain a high density of –COOH groups that bind lysozyme through ion-exchange. **Table 3.3** and **Figure 3.5** show results for lysozyme binding to a control electrospun membrane, the modified electrospun membrane, and a modified PET membrane support. The breakthrough curves in **Figure 3.4** clearly show the most extensive binding to the PAA/PEI/PAA-modified nylon fiber membrane, and the lysozyme binding capacity in this membrane is 115 mg/cm³ of membrane (the binding capacity was calculated only using the thickness of the nylon fibers which is 30μm), similar to values for traditional nylon membranes with 1.2 μm pores (1). Moreover, the low binding capacities for the unmodified membrane and the modified PET support indicate that the binding mainly occurs to the PAA/PEI/PAA films on the electrospun fibers.

Table 3.3. Lysozyme binding capacities of electrospun nylon fiber membranes with and without PAA/PEI/PAA films and of a PAA/PEI/PAA-modified PET support that was detached from the nylon membrane.

	unmodified membrane	PAA/PEI/PAA-modified membrane	PAA/PEI/PAA-modified PET layer
binding capacity (mg/mL)	4	115±5*	7

*The ± value represents the standard deviation of binding capacities for three different membranes.

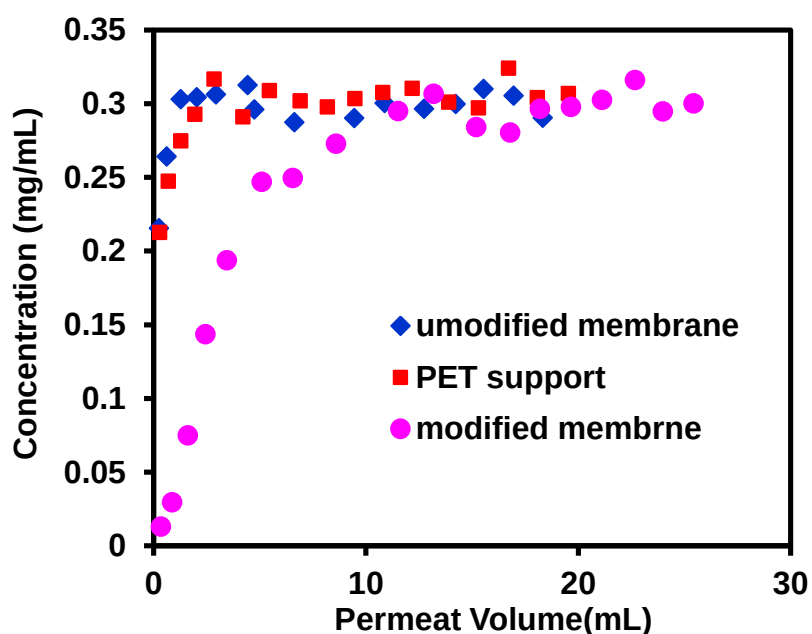


Figure 3.5. Lysozyme breakthrough curves during passage of 0.3 mg/mL of lysozyme through PET-supported nylon fiber membranes with and without modification by a PAA/PEI/PAA film and through a PAA/PEI/PAA-modified PET support. The PAA/PEI/PAA films were deposited at pH 3 from 0.02 M PAA solutions containing 0.5 M NaCl and 2 mg/mL PEI solutions. The lysozyme feed solution contained 20 mM phosphate buffer at pH 7.4.

3.3.5.2. Con A binding

Derivatization of the electrospun fibers with PAA/PEI/PAA-NTA-Cu²⁺ allows capture of Con A, a protein that has 4 subunits, each of which contains 6 histidine residues. These residues interact with Cu²⁺ ions loaded in the membrane. The nylon fiber membrane gave a Con A binding capacity of 86±12 mg/cm³ of membrane (**Figure 3.6**). Again this is similar to the binding capacity for traditional spongy nylon membranes (pore diameter of 1.2 μm) modified with PAA/PEI/PAA-NTA-Cu²⁺ (1).

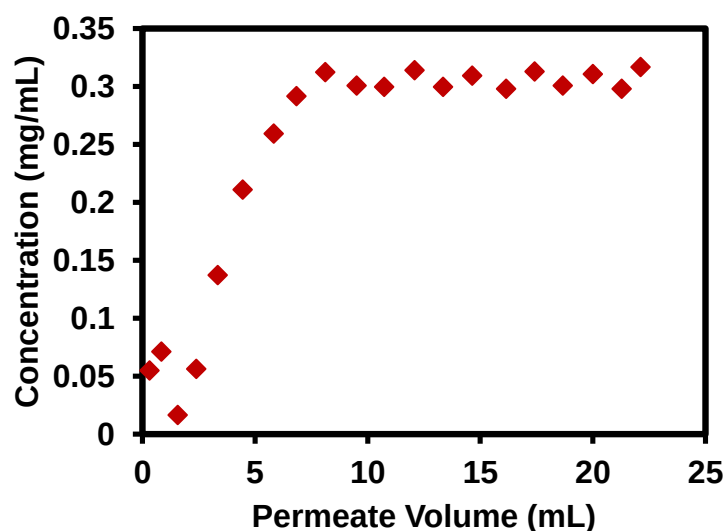


Figure 3.6. Breakthrough curve during passage of 0.3 mg/mL Con A through an electrospun nylon membrane modified with PAA/PEI/PAA-NTA-Cu²⁺. The binding solution contained 20 mM phosphate buffer at pH 6.

3.3.5.3 Challenges with electrospun membranes

Unfortunately, large flux drops occurred after modification of the electrospun fibers (**Table 3.4**). Compared to a bare membrane, the adsorption of polyelectrolytes decreased the water flux 80%. This flux drop mainly stems from modification of the membrane fibers rather than modification of the PET support layer, as the flux through the modified PET support still has high flux after modification. Scanning electron microscope (SEM) images (**Figure 3.7**) suggest the high flux drop in modified membranes results from PEMs that span gaps between fibers. Even worse, membranes modified with PAA/PEI/PAA-NTA-Cu²⁺ are almost impermeable. The flow rate dropped to about 0.1 mL/min when passing a Con A solution through such membranes using a peristaltic pump.

Table 3.4. Water permeabilities of an unmodified electrospun nylon fiber membrane and of similar membranes after modification with PAA/PEI/PAA and PAA/PEI/PAA-NTA-Cu²⁺ films. (Buffer was not passed through the substrates before flux measurements). The table also shows the permeability of a PAA/PEI/PAA-modified PET electrospinning support. The pressure was 0.68 atm and the unit for permeability is mL/(cm² min atm). The $\pm$ value represents the standard deviation of values for three different membranes.

	Unmodified electrospun membrane	PAA/PEI/PAA -modified PET support	PAA/PEI/PAA-modified electrospun membrane	PAA/PEI/PAA-NTA-Cu²⁺-modified electrospun membrane
flux	63.7$\pm$1.4	56.2$\pm$0.9	14.1$\pm$0.5	0.35$\pm$0.09

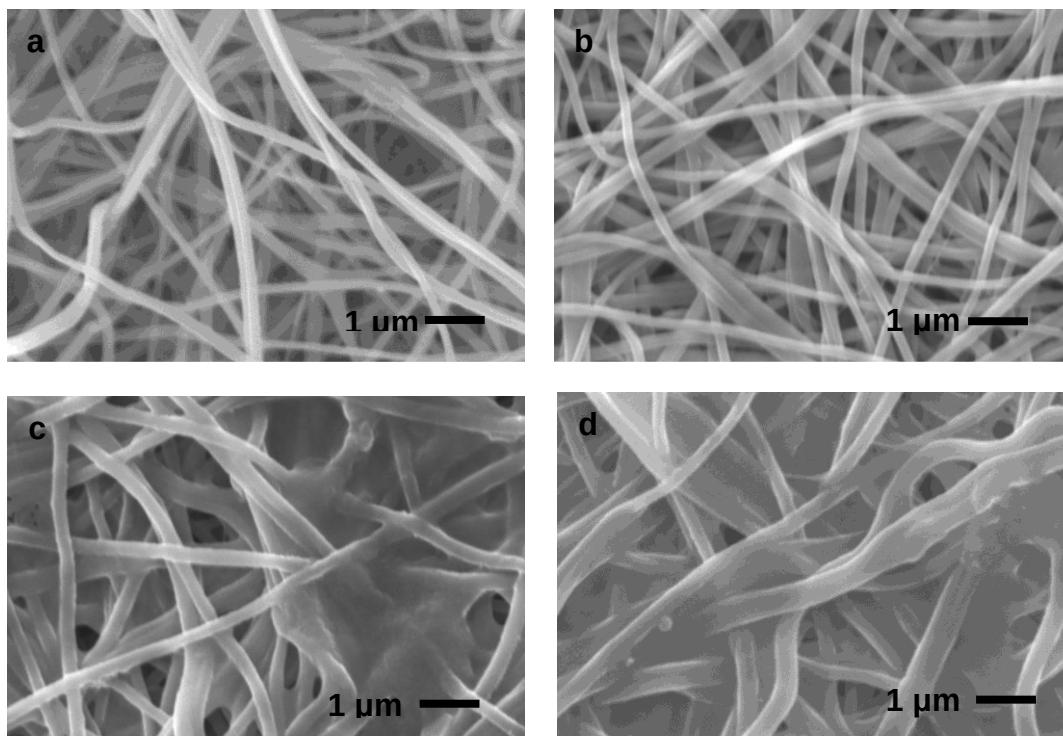


Figure 3.7. SEM images of electrospun nylon fiber membranes: (a) an unmodified membrane, (b) an unmodified membrane after water flux measurements, (c) a membrane modified with a PAA/PEI/PAA film and (d) a membrane modified with PAA/PEI/PAA-NTA-Cu²⁺.

3.3.6. Membrane modification with PNTA

Modification of nylon membranes with PAA/PEI/PAA-NTA-Ni²⁺ provides membranes with both high capacity and high selectivity for binding His-tagged proteins (see above). However, the derivatization of these films with aminobutyl NTA is both inefficient and expensive. The reagents for this derivatization represent more than 95% of the membrane cost, and most of the aminobutyl NTA does not bind to the membrane. To simplify the membrane fabrication, Salinda Wijeratne synthesized PNTA, which contains the NTA functionality and can also directly participate in layer-by-layer

adsorption to avoid the derivatization step (**Figure 3.8**).

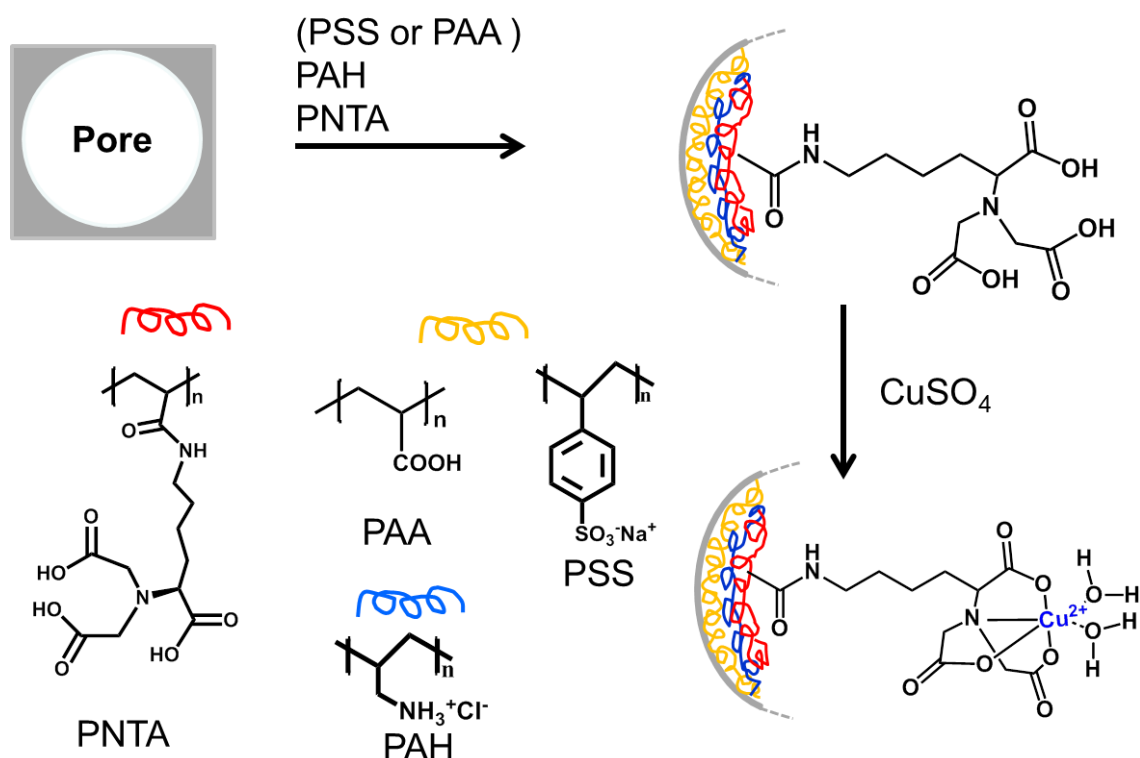


Figure 3.8. Membrane modification with PNTA- Cu^{2+} -containing films.

Because PNTA is a new polymer for LbL deposition, we examined a variety of conditions for membrane modification. Based on our previous work (1), we chose PNTA, PSS, and PAA as initial layers for subsequent growth of PAH/PNTA films. Both PSS and PAA adhere well to nylon, probably through hydrophobic interactions, and are effective priming layers. However, an initial layer of PNTA may enhance metal-ion and protein binding. **Table 3.5** shows the Cu^{2+} -binding capacities in membranes with PNTA-containing films deposited under different conditions. The quantity of Cu^{2+} bound by the membranes serves as an indicator of the amount of adsorbed PNTA. We first directly deposited PNTA-containing films in the membrane using 1 mg/mL PNTA and PAH solutions at pH 3 with no NaCl in the deposition solution. PEI was not chosen as

the polycation because it also binds Cu^{2+} to interfere Cu^{2+} binding on PNTA (28, 29). The Cu^{2+} -binding capacity for these PNTA/PAH/PNTA- and PNTA(PAH/PNTA)₂-modified membranes is <2 mg/mL (Table 3.5, entries 1 and 2). Moreover, addition of 0.5 M NaCl to polyelectrolyte adsorption solutions did not increase the Cu^{2+} binding capacities even with PNTA/PAH/PNTA and PNTA(PAH/PNTA)₂ films (Table 3.5, entry 3). In contrast, PAA/PAH/PAA-NTA-modified membranes (films grown with 0.5 M NaCl) capture 14 mg of $\text{Cu}^{2+}/\text{cm}^3$ of membrane (1). The low Cu^{2+} -binding capacity for films containing PNTA suggests limited polyelectrolyte adsorption in membrane pores. Thus, we developed strategies to increase the film growth.

Table 3.5. Cu^{2+} binding capacities of nylon membranes (1.2 μm pores) modified with PNTA-containing films deposited under different conditions.

Entry	Deposition conditions	Film	Cu^{2+} binding capacity (mg/cm ³)
1	pH 3.0 for PNTA and PAH, no NaCl	PNTA/PAH/PNTA	1.2
2		PNTA(PAH/PNTA) ₂	1.5
3	pH 3.0 for PNTA and PAH, 0.5 M NaCl	PNTA(PAH/PNTA) ₂	1.4
4	pH 3.0 for PNTA and PSS, pH 9 for PAH, no NaCl	PNTA/PAH/PNTA	1.5
5		PNTA(PAH/PNTA) ₂	1.8
6		PSS/PAH/PNTA	1.0
7		PSS(PAH/PNTA) ₂	3.8
8	pH 3 for PNTA, PSS and/or PAA, pH 9 for PAH, 0.5 M NaCl	PSS/PAH/PNTA	1.9
9		PSS(PAH/PNTA) ₂	9.4
10		PAA/PAH/PNTA	8.4
11		PAA(PAH/PNTA) ₂	19.0
12		PAA/PAH	2.1

As discussed in chapter 2, pH greatly affects the adsorption of weak polyelectrolytes such as PNTA and PAH. Adsorption of PAH at pH 9 rather than 3 increased the Cu^{2+} binding to PNTA/PAH/PNTA- and PNTA(PAH/PNTA)₂-modified membranes (**Table 3.5, entries 4 and 5**) about 30%. The high deposition pH increases PAH adsorption, either by decreasing the charge on PAH or increasing the charge on already adsorbed PNTA. Either mechanism will require adsorption of more PAH to compensate the surface charge. Thus all subsequent experiments employed pH 3 for PNTA adsorption and pH 9 for PAH adsorption.

Our previous work suggests that PSS adsorbs strongly to nylon membranes (30). Thus, in some experiments we employed PSS as an adhesion layer for subsequent adsorption of PAH/PNTA. For deposition in the absence of salt, PSS/PAH/PNTA films give lower Cu^{2+} -binding capacities than PNTA/PAH/PNTA films (compare **entries 4 and 6 in Table 3.5**), likely because the inner PNTA layer binds Cu^{2+} and PSS does not. However, addition of a second PAH/PNTA bilayer leads to twice as much binding for the PSS-initiated film relative to the PNTA(PAH/PNTA)₂ film (compare **entries 5 and 7 in Table 3.5**). The initial PSS layer probably templates more polyelectrolyte adsorption than a first layer of PNTA.

Salt added to deposition solutions screens the charges on polyelectrolytes to enhance their deposition. Addition of 0.5 M NaCl to all polyelectrolyte deposition solutions increases Cu^{2+} binding by a factor of 1.9 (compare **entries 6 and 8 in Table 3.5**) and 2.5 (compare **entries 7 and 9 in Table 3.5**) for PSS(PAH/PNTA) and PSS(PAH/PNTA)₂ films, respectively. Finally, we examined PAA as the initial layer using deposition from

salt-containing solutions. This clearly leads to the highest Cu^{2+} binding with values reaching 19.4 mg/cm^3 for $\text{PAA}/(\text{PAH}/\text{PNTA})_2$ films. Because free $-\text{COOH}$ groups in PAA might be responsible for some of this binding, we also determined the Cu^{2+} capture by membranes modified with a PAA/PAH film. Such membranes bind only $2.1 \text{ mg Cu}^{2+}/\text{cm}^3$ of membrane (compare **entries 11 and 12 in Table 3.5**). Even if we take this adsorption into account, the use of PAA as an initial layer in nylon membranes clearly increases Cu^{2+} capture.

For His-tagged protein capture both the quantity of metal-ion binding and the strength of binding are important, as weakly bound ions may simply leach from the membrane. **Figure 3.9**, shows the Cu^{2+} leaching in the buffers we employ for protein binding, washing, elution, and metal-ion stripping. These studies examined membranes containing $\text{PSS}(\text{PAH}/\text{PNTA})_2$ and $\text{PAA}(\text{PAH}/\text{PNTA})_2$ deposited from solutions containing 0.5 M NaCl because they have high metal-ion binding capacities. As **Figure 3.9** shows, in binding and washing buffers, the PNTA-containing membranes exhibits much less Cu^{2+} leaching than $\text{PAA}/\text{PEI}/\text{PAA}$ -NTA-modified membranes. The $\text{PAA}/\text{PEI}/\text{PAA}$ -NTA membranes lose nearly 20% of their Cu^{2+} in a binding buffer containing 10 mM imidazole . Much of this leaching likely includes Cu^{2+} bound to underivatized free $-\text{COO}^-$ groups of PAA. After subsequent washes with washing buffers I and II, only half of the Cu^{2+} remains in the $\text{PAA}/\text{PEI}/\text{PAA}$ -NTA-modified membrane. The PNTA-containing films are more resistant to leaching, even when the films have PAA as the first layer. For these films, during adsorption of PAH at pH 9, $-\text{COOH}$ groups in PAA will deprotonate and form ion pairs with ammonium groups of PAA.

Such groups likely do not bind significant amounts of metal ions, and Cu^{2+} capture occurs primarily through NTA groups.

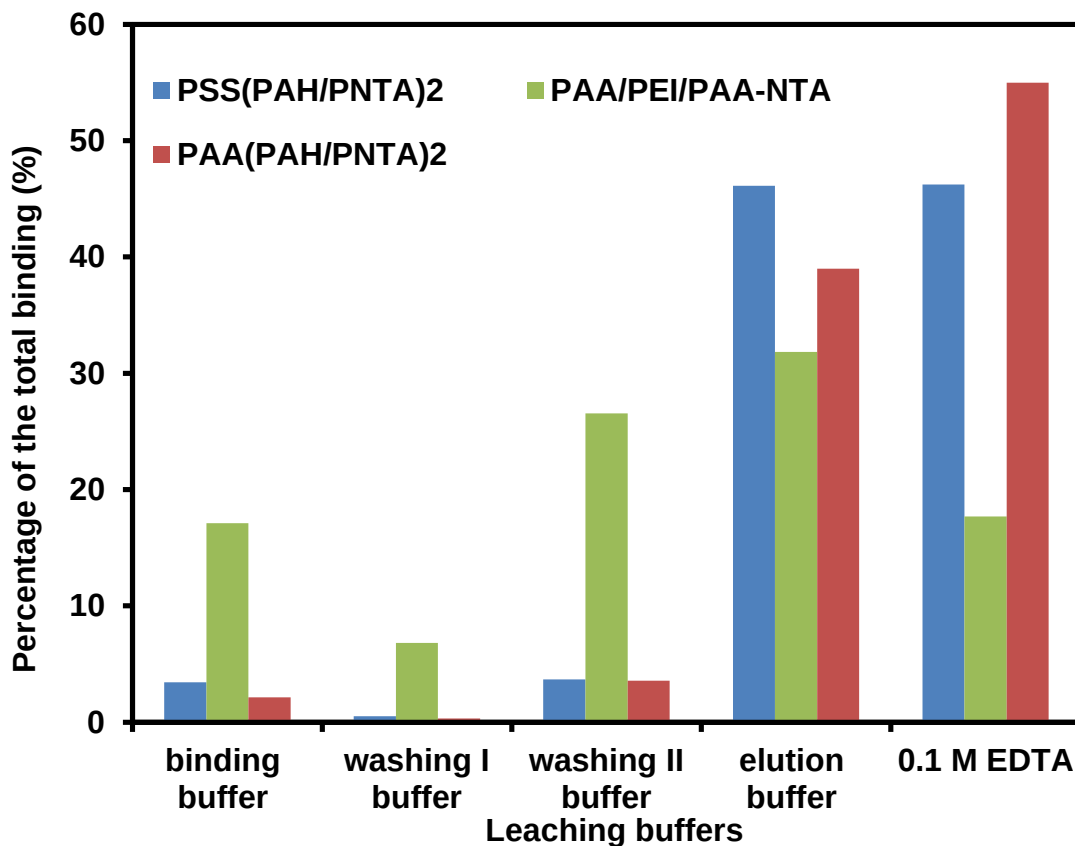


Figure 3.9. Percentage of the bound Cu^{2+} leached from PSS(PAH/PNTA)₂-, PAA(PAH/PNTA)₂- and PAA/PEI/PAA-NTA-modified membranes when passing 5 mL of buffers sequentially through the membranes. Deposition conditions for PSS(PAH/PNTA)₂, PAA(PAH/PNTA)₂ are those in footnote c in Table 3.4. PAA/PEI/PAA-NTA was deposited as described in section 3.2.2.

For all films, extensive leaching occurs in the 0.5 M imidazole solution that elutes proteins. At high concentration, imidazole effectively competes with NTA for Cu^{2+} ions and leaching occurs. Still, the PNTA-containing membranes retain half of their Cu^{2+} even after passing 0.5 M imidazole through the membrane. Importantly, His-tagged

protein purification will employ Ni^{2+} rather than Cu^{2+} because Ni^{2+} has a lower affinity for imidazole, which should lead to less non-specific adsorption of proteins that contain histidine residues and not polyhistidine. The relatively low affinity of imidazole for Ni^{2+} should also decrease leaching in the elution buffer.

3.4. Conclusions

PAA/PEI/PAA multilayers provide a platform for creating derivatizable membranes. When deposited from pH 3 solutions, PAA/PEI/PAA films swell extensively at neutral pH because of a high density of free -COO- groups. These groups are also available for further modification with aminobutyl NTA, which chelates Cu^{2+} or Ni^{2+} ions. Membranes modified with PAA/PEI/PAA-NTA- Ni^{2+} enable rapid isolation of His-tagged proteins from cell extracts with > 95% purity and recovery. Spongy nylon membranes are excellent substrates for PEM modification, so we also examined whether modification of highly porous mats of electrospun nylon fibers could create highly permeable membranes with high protein-binding capacities. However, the flux through the electrospun membranes declined dramatically after modification.

Membranes with PAA/PEI/PAA-NTA- Ni^{2+} films successfully isolate His-tagged protein, but metal-ion leaching is extensive. A newly designed polymer that contains NTA groups, PNTA, greatly simplifies formation of membranes with chelating groups, and importantly the membranes show less Cu^{2+} leaching than PAA/PEI/PAA-NTA. In the future, we will examine the PNTA-modified membranes for Ni^{2+} binding and His-tagged protein purification.

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Chapter 4. Enrichment of N-Glycoproteins Using Membranes

Modified with Lectins

Chapter 3 described our development of membranes for His-tagged protein purification. This chapter presents my efforts to create membranes that capture N-glycoproteins via binding of glycans to an immobilized lectin, Concanavalin A (Con A). Thus, I immobilized Con A in membranes using covalent or metal-affinity methods with either polymer brushes or LbL films.

4.1. Introduction

Post-translational modifications (PTMs) of proteins are chemical modifications that occur on specific amino acids after protein expression (2, 3). These PTMs can affect protein structures and regulate catalytic activities, localization, and cell signaling (2, 4-7). In the human genome, DNA only encodes the 20 essential amino acids (8), but PTMs enhance the range of protein functions, particularly in response to cellular development requirements or changes in the physiological environment (2, 8). There are more than 200 types of PTMs and over 80000 specific PTMs have been characterized experimentally (9-11).

Glycosylation, the attachment of sugar moieties (glycans) to proteins, is one of the most common PTMs in eukaryotes (12). Protein glycosylation can be divided into two types: N-glycosylation and O-glycosylation, based on the glycosylation sites and the type of linkage between the protein and the glycans (13-15). In N-glycosylated proteins, amide linkages connect *N*-acetylglucosamines to Asparagine (Asn) residues that must

reside in consensus sequences containing Asn-any amino acid-Serine/Threonine (15). With O-glycosylated proteins, ether linkages attach glycans to Serine or Threonine (16). A complex series of enzymes catalyze glycan synthesis and their attachment to proteins (17). In the endoplasmatic reticulum, N-glycosylation on Asn employs more than 23 enzymatic steps in assembly, trimming, and maturation of the carbohydrate groups (18). Many factors, especially the cell stage affect this complicated process and increase the heterogeneity of the glycans. For example, at least 5 different glycan structures appear at ⁶⁰Asn in bovine Ribonuclease B (19).

Several studies report that N-glycans play a role in physiological and pathological activities including cell adhesion, migration, signaling, differentiation, and tumor invasion (20-22). Some aberrant glycans reflect the development and progress of cancers. For example, E-cadherin, a cell surface protein primarily found in neural tissues and fibroblasts, is the main epithelial cell-cell adhesion molecule involved in Ca²⁺-dependent cell-cell adhesion associated with cancer metastasis. Attachment of *N*-acetylglucosamine to a branching position on the N-glycans connected to E-cadherin promotes cancer progression and metastasis. In contrast, N-glycans with a bisecting conformation may suppress the cancer metastasis (23). Therefore, glycoproteins are a promising source of biomarkers for cancers, including CA125 for ovarian cancer (24) and Her2/neu for breast cancer (25).

Despite advances in glycoprotein research, exploration of glycosylation on a proteome scale is challenging due to the low abundance and heterogeneities of the glycans. In vivo, less than 1% of total proteins are glycosylated (2), so purification of

glycoproteins is vital to their identification. Currently, lectin affinity chromatography is the most common method for enriching glycoproteins (26). Lectin capture in membranes may simplify glycoprotein isolation due to the advantage of convective transport to binding sites. Thus in this work, I aimed to develop a Con A-containing membrane for rapid glycoprotein purification. This chapter investigates different strategies for Con A immobilization and subsequent glycoprotein binding to immobilized Con A.

4.2. Experimental section

4.2.1. Materials

Hydroxylated nylon (LoProdyne® LP, Pall, 1.2 μm pore size, 110 μm thick) was cut into 25 mm-diameter discs prior to use. Coomassie protein assay reagent (Thermo Scientific), Tris(2-(dimethylamino)ethyl)amine (Me_6TREN , ATRP Solutions) and other chemicals from Sigma-Aldrich were used as received. The trichlorosilane initiator (11-(2-bromo-2-methyl)propionyloxy)-undecyltrichlorosilane) and macroinitiator ((poly(2-(trimethylammonium iodide)ethyl methacrylate-co-2-(2-bromoisobutyryloxy)ethyl acrylate)) were synthesized according to literature procedures (27-29). Buffers were prepared using analytical grade chemicals and deionized (Milli-Q, 18.2 $\text{M}\Omega\text{ cm}$) water.

4.2.2. Initiator adsorption

4.2.2.1. Trichlorosilane initiator

Before the attachment of initiator, the hydroxylated nylon membrane was cleaned

with UV/ozone for 15 min. Initiator attachment occurred by circulating 1 mM trichlorosilane initiator in anhydrous tetrahydrofuran through the membrane for 2 h at a flow rate of approximately 3 mL/min using a peristaltic pump. This was followed by sequentially passing 20 mL of tetrahydrofuran, 20 mL of ethanol, and 20 mL of water through the membrane before drying with a N₂ stream.

4.2.2.2. Macroinitiator

The UV/ozone-cleaned membrane was modified by flowing 10 mL of 2 mg/mL aqueous macroinitiator through the membrane for 10 min at a flow rate of 1 mL/min using a peristaltic pump (30). Subsequently, 20 mL of water was passed through the membrane, which was then dried and stored for polymerization.

4.2.3. .Preparation of catalyst and monomer solutions

Atom transfer radical polymerization (ATRP) was performed with a Cu (I)/Cu(II) catalyst (31). For a 20-mL catalyst solution, 0.0447 g of CuBr₂ was dissolved in 20 mL of *N,N*-dimethylformamide (DMF) in a round-bottom flask and the solution was degassed using 2 freeze-pump-thaw (FPT) cycles on a Schlenk line. Subsequently, 0.184 mL of Me₆TREN was added to the mixture of CuBr₂ and DMF using a syringe. Two more FPT cycles were applied immediately to degass the solution. Subsequently, 0.0574 g CuBr was added, and the solution was degassed using 3 FPT cycles. In a glove bag, the catalyst solution was transferred into 20 mL vials and stored there at room temperature for no more than a week. The monomer solution, which contained 2-(methacryloyloxy)ethyl succinate (MES) mixed with 1 M aqueous NaOH (1:1, v/v),

was degassed using FPT cycles before polymerization just prior to polymerization (30).

4.2.4. Membrane modification with polymeric films

To prepare polymerization solutions, 10 mL of freshly degassed monomer solution was mixed with 1 mL of catalyst solution in a glove bag. The catalyst concentration in the mixture was CuBr₂ (1 mM), CuBr (2 mM) and Me₆TREN (6 mM). Still in the glove bag, the monomer/catalyst solution was circulated through an initiator-modified membrane using a peristaltic pump at a flow rate of 1 mL/min. After the desired polymerization time, 20 mL of ethanol and 20 mL of water were passed sequentially through the membrane at the same flow rate. PAA/PEI/PAA-modified membranes were also employed for Con A immobilization, and these membranes were prepared as described in section 3.2.2 of chapter 3 and treated the same as membranes containing poly(MES) for derivatization. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Perkin Elmer Spectrum One Instrument, air background) was employed to verify the modifications.

4.2.5. Derivatization of polymeric films with Con A

4.2.5.1. Cu²⁺-affinity binding

The poly(MES) in the membrane was activated by passing an aqueous solution of 0.05 M *N*-hydroxysuccinimide (NHS)/0.05 M 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) through the membrane for 1 h at 1 mL/min. The membrane was then washed with 20 mL of water

and 20 mL of ethanol. After activation, an aqueous solution of 0.1 M N_{α},N_{α} -bis(carboxymethyl)-L-Lysine hydrate (aminobutyl NTA, pH 10) was circulated through NHS-modified membrane for 1 h at a flow rate of 1 mL/min followed by a 20-mL water wash. Then 20 mL of 0.1 M aqueous CuSO_4 was circulated through the membrane for 2 hours at the same flow rate followed by washing with 20 mL water. Con A was immobilized to the poly(MES)-NTA- Cu^{2+} -modified membrane by circulating a 0.3 mg/mL Con A solution in 20 mM phosphate buffer (pH 6) through the membrane for 1 h. Excess Cu^{2+} sites were blocked with bovine serum albumin (BSA, which does not bind to Con A) by subsequently circulating a 0.3 mg/mL BSA solution in phosphate buffer (20 mM, pH 7.4) through the membrane for 15 min (32). The amount of Con A immobilized on polymer brushes was estimated using a protein Bradford assay to determine the amount of Con A in the loading solution before and after circulating through the membrane. The membranes were dried and stored for glycoprotein binding tests.

4.2.5.2. Covalent derivatization

Similarly to Cu^{2+} -affinity immobilization, the first step is to activate the carboxylic acid groups on the polymer brushes with 0.05 M NHS/0.05 M EDC for 1 hour. After rinsing as described above, 3 mL of 0.3 mg/mL Con A solution in 20 mM phosphate buffer (pH 6) was circulated through the membrane for 4 hours at a flow rate of 1 mL/min followed by 40 mL of phosphate buffer (pH 6). The amount of Con A immobilized on the polymer brushes was calculated as described in section 4.2.5.1. The

membranes were dried with N₂ and stored for further analysis.

4.2.5.3. Immobilization of protected Con A

To preserve the glycan binding sites, Con A was protected with methyl- α -D-mannopyranoside by incubating 0.3 mg/mL Con A in 20 mM Tris buffer (pH 6) containing 500 mM NaCl, 5mM MgCl₂, 5 mM MnCl₂, 5 mM CaCl₂ and 200 mM methyl- α -D-mannopyranoside for 30 min. After membrane activation as described above, this Con A solution (containing all the reagents including the protecting agent) was employed to anchor Con A to the NHS-activated membrane and the Cu²⁺-membrane in the same way as described in sections 4.2.5.1 and 4.2.5.2. After immobilization, the Con A was regenerated by washing the membrane with 3 mL of 100 mM Tris buffer supplemented with 500 mM NaCl (pH 8.5) followed by 3 mL of 100 mM sodium acetate with 1.0 M NaCl (pH 4.5). This process was performed three times. Prior to tests of glycoprotein binding, the membranes were equilibrated with 20 mM phosphate buffer at pH 7.4 (33).

4.2.6. Measurement of protein binding capacities

Bradford Assays were also used to determine protein-binding capacities from breakthrough curves and protein elutions. In this study, lysozyme (in 20 M phosphate buffer, pH 7.4), Con A (in 20 mM phosphate buffer, pH 6) and Asialofetuin (in 20 mM Tris, 0.15 M NaCl, 1 mM MnCl₂, and 1 mM CaCl₂, pH 7.4) were pumped through membranes at a flow rate of about 1 mL/min. The permeate solution was collected at specific time intervals, and 40 mL of phosphate or Tris buffers were used to rinse the

membrane before elution. Then, lysozyme was eluted with 20 mM phosphate buffer containing 1 M KSCN (pH 7.4), Cu²⁺-chelated Con A was eluted with 20 mM phosphate buffer containing 0.1 M ethylenediamine tetraacetic acid (EDTA, pH 6) and asialofetuin was eluted with 20 mM Tris buffer containing 0.5 M methyl- α -D-mannopyranoside (pH 7.4). To determine effluent and eluate concentrations, a series of standard solutions with concentrations ranging from 0 to the feed concentration for each protein were prepared and 30 μ L of protein solution including the standards and samples was diluted into 1.5 mL Coomassie Brilliant Blue solution (used as received) at room temperature (34). The absorbance of these solutions was determined using a Perkin-Elmer UV/vis (model Lambda 40) spectrophotometer at 595 nm.

4.3. Results and Discussion

4.3.1. Membrane modification with poly(MES) films

The first step in creating lectin-based glycoprotein-binding membranes is lectin immobilization. Anchoring through poly(MES) brushes may yield multilayers of immobilized lectin (27, 30, 34, 35) to give a high capacity in subsequent capture of glycoproteins. Growth of poly(MES) in membranes begins with initiator attachment, and **Figure 4.1** shows the strategies we employ to introduce the initiating groups. The trichlorosilane initiator attaches to the hydroxylated nylon membrane via Si-O bonds (**Figure 4.1a**). We polymerized MES from the immobilized trichlorosilane initiator for 1 hour, 30 min and 15 min to see if more polymerization would increase protein binding. However, after 1 hour of polymerization, the polymer clogged the membrane pores. In

initial studies of protein capture, we examined lysozyme binding to poly(MES) via ion exchange. Based on breakthrough curves (**Figure 4.2**), the 30-min polymerization and 15-min polymerization give similar binding amounts of 85-90 mg/cm³. Note that this is not the saturation binding capacity, as the effluent lysozyme concentration is still less than that in the feed at the end of loading. Unfortunately, the flux through the membranes modified with 30 min of polymerization membrane declined severely during the lysozyme binding studies. Even with the 15-min polymerization the permeability declined from 115 mL/(cm² min atm) (bare membrane) to 2.9 mL/(cm² min atm) after lysozyme binding. In our previous studies, we also noticed flux drop when using the trichlorosilane initiator (35).

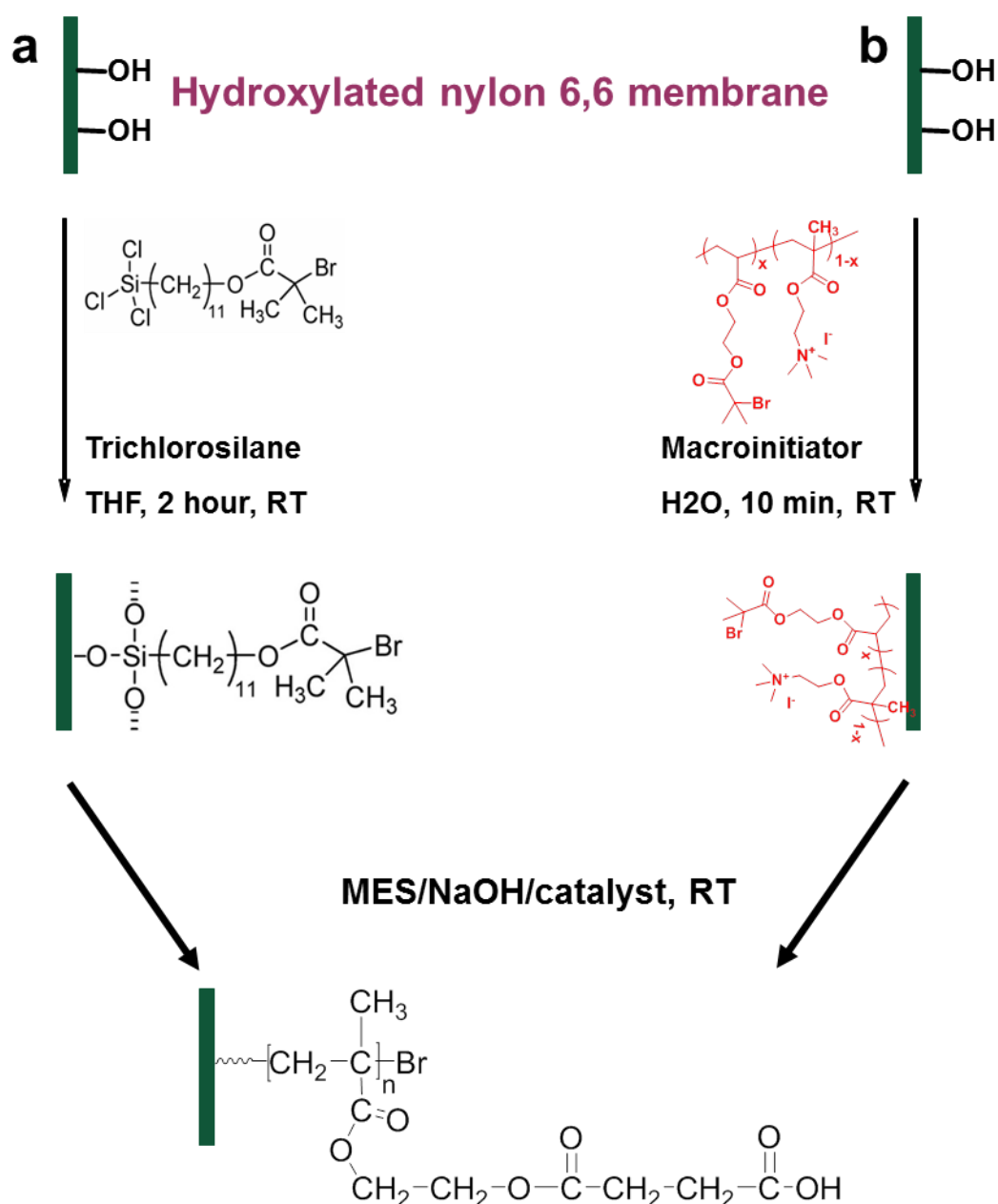


Figure 4.1. Growth of poly(MES) brushes from nylon membranes using (a) a trichlorosilane initiator and (b) a macroinitiator. MES was polymerized using a Cu(I)/Cu(II)/Me₆TREN/DMF catalyst at room temperature.

To increase permeability, we employed a macroinitiator to initiate polymerization in the membranes. The macroinitiator attaches to the pore surface via hydrophobic interactions (**Figure 4.1b**) and likely gives less dense polymer brushes than initiation

from the trichlorosilane initiator. Using the macroinitiator, Anuraj and coworkers found that lysozyme binding capacity increased as the following MES polymerization time increased from 2 to 5 min but decreased for longer polymerization, with a corresponding flux decreased as a function of time (30). Thus, we use only the 5-min polymerization time for the macroinitiator in this chapter, and under those conditions, the poly(MES) binds around 107 mg lysozyme/cm³ of membrane (**Figure 4.2**), although again this is not the saturation binding capacity. Importantly the flux was still above 35 mL/(cm² min atm) after protein sorption. In addition, the macroinitiator interacts adsorbs to the membrane from aqueous solution in only 10 min and thus is easier to apply than the trichlorosilane initiator. Thus, the following discussion focuses on brushes grown from macroinitiator-modified nylon membranes.

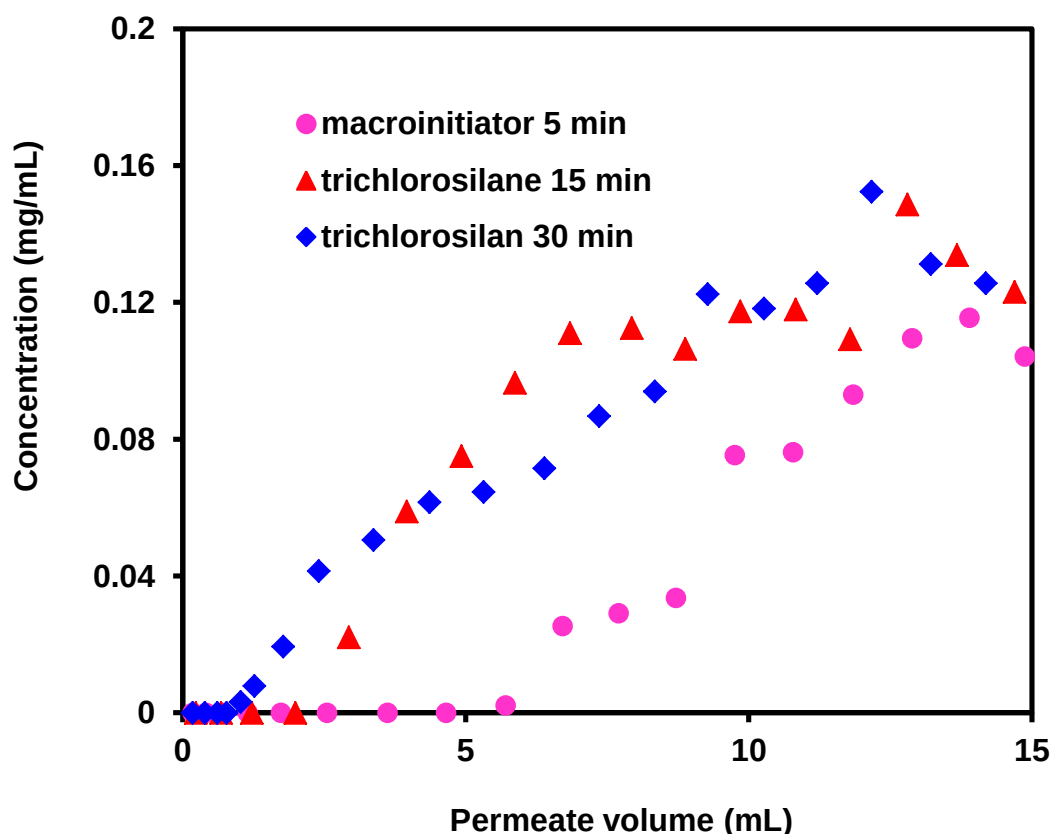


Figure 4.2. Lysozyme breakthrough curves during passage of a 0.3 mg/mL lysozyme solution through nylon membranes modified with poly(MES) brushes grown from trichlorosilane initiator for 30 or 15 min or macroinitiator for 5 min.

All lysozyme feed solutions contained 20 mM phosphate buffer (pH 7.4). Flow rates were around 1 mL/min through the membranes (area of 3.1 cm²) modified using the macroinitiator or the 15-min polymerization from trichlorosilane initiator, but much smaller with the membrane modified with 30-min of polymerization from the trichlorosilane initiator because the peristaltic pump could not maintain the flow rate.

4.3.2. Con A immobilization via Cu²⁺-affinity and subsequent glycoprotein binding

Con A contains four identical subunits, each of which has a binding site for

glycoprotein, and is the most common lectin for N-glycoprotein binding (**Figure 4.3**) (1, 36-38). The presence of Mn^{2+} and Ca^{2+} in the N-glycoprotein binding buffer is crucial to maintain Con A's binding activity. With regard to immobilization, each Con A subunit contains six histidine residues that could interact with Cu^{2+} on a solid support (39).

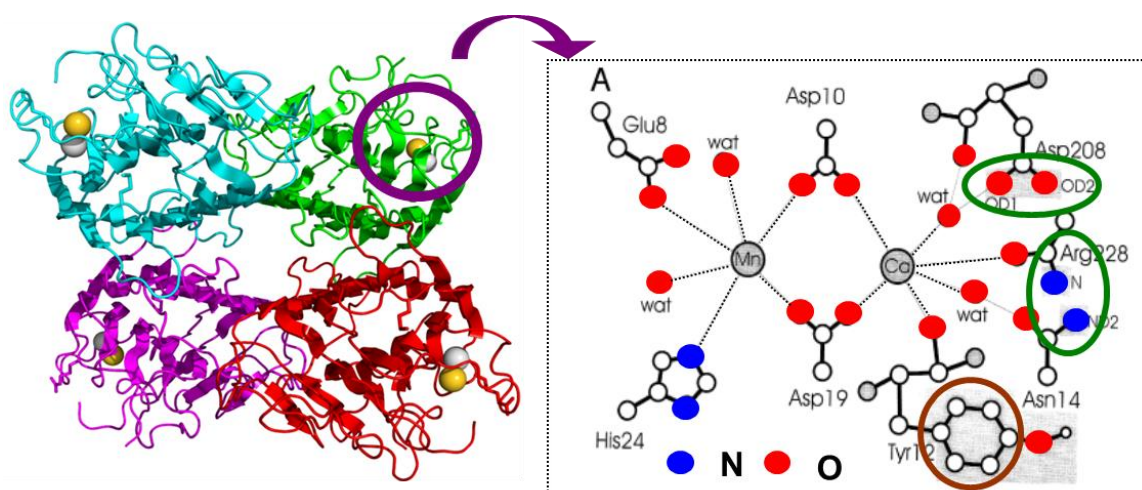


Figure 4.3. Conformation of Con A (left) and illustration of its glycoprotein binding sites. Each subunit contains Mn^{2+} and Ca^{2+} binding sites that help maintain the active conformation. A series of hydrogen bonding on ^{208}Asp , ^{228}Arg and ^{14}Asn (circled in green) and hydrophobic interactions with ^{12}Try (circled in brown) lead to the glycan (1). Redrawn from reference 1 and 38 with permission of Elsevier and the American Chemical Society, respectively.

In one method to immobilize Con A, we formed Cu^{2+} -NTA complexes in the membrane by first attaching aminobutyl NTA to the polymer brushes with NHS/EDC coupling as described in chapter 3. We monitored the reaction with aminobutyl NTA using ATR-IR spectroscopy (**Figure 4.4**). Bare nylon membranes have amide I and II absorbance peaks at 1630 and 1533 cm^{-1} , respectively (**Figure 4.4, spectrum a**). After

polymerization, the new peak at 1720 cm^{-1} (**Figure 4.4, spectrum b**) arises from the acid carbonyl group on poly(MES). To functionalize the polymer brushes with NTA, the --COOH groups of poly(MES) were firstly activated by NHS. The peaks at 1810 and 1770 cm^{-1} due to newly formed succinimidyl ester groups verify the activation (**Figure 4.4, spectrum c**). After reaction with NTA, the absorbances from the succinimidyl ester disappeared. However, the ATR-IR spectra of the NTA- and NTA-Cu^{2+} -functionalized membrane (**Figure 4.4, spectra d and e**), do not show any readily apparent new absorbances, presumably because they are masked by the strong amide absorbances of nylon. These amide absorbances also preclude detection of protein binding through ATR-IR spectroscopy. Therefore, we determined the Con A binding using a breakthrough curve (**Figure 4.5**), which indicated a Con A binding capacity of about 69 mg/cm^3 of membrane.

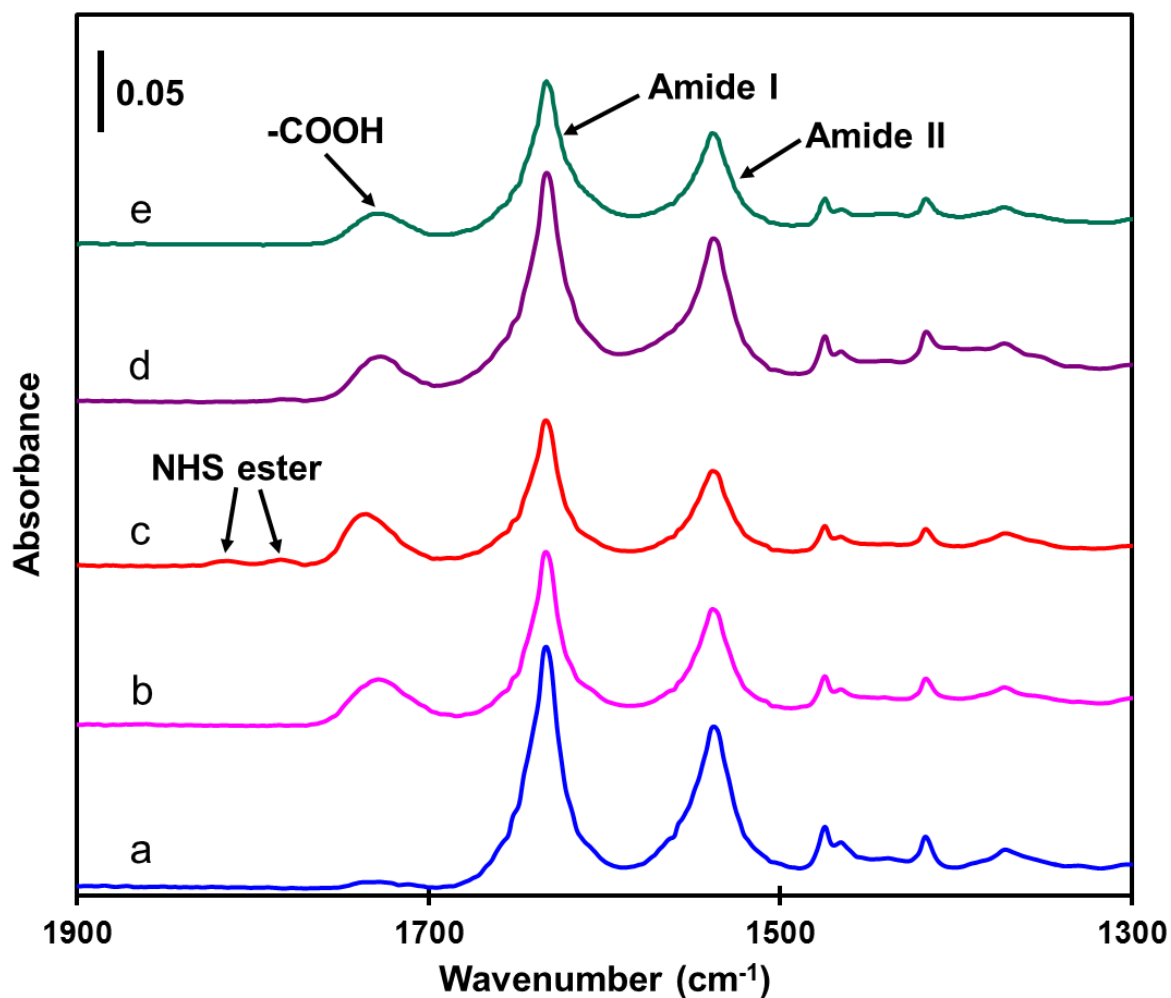


Figure 4.4. ATR-IR spectra of nylon membranes before and after steps for membrane functionalization. (a) Nylon membrane; (b) Nylon membrane-poly(MES); (c) Nylon membrane-poly(MES)-NHS; (d) Nylon membrane-poly(MES)-NTA; (e) Nylon membrane -poly(MES)-NTA-Cu²⁺. The membranes were washed with 20 mM phosphate buffer (pH 7.4) followed by deionized water prior to obtaining the spectra.

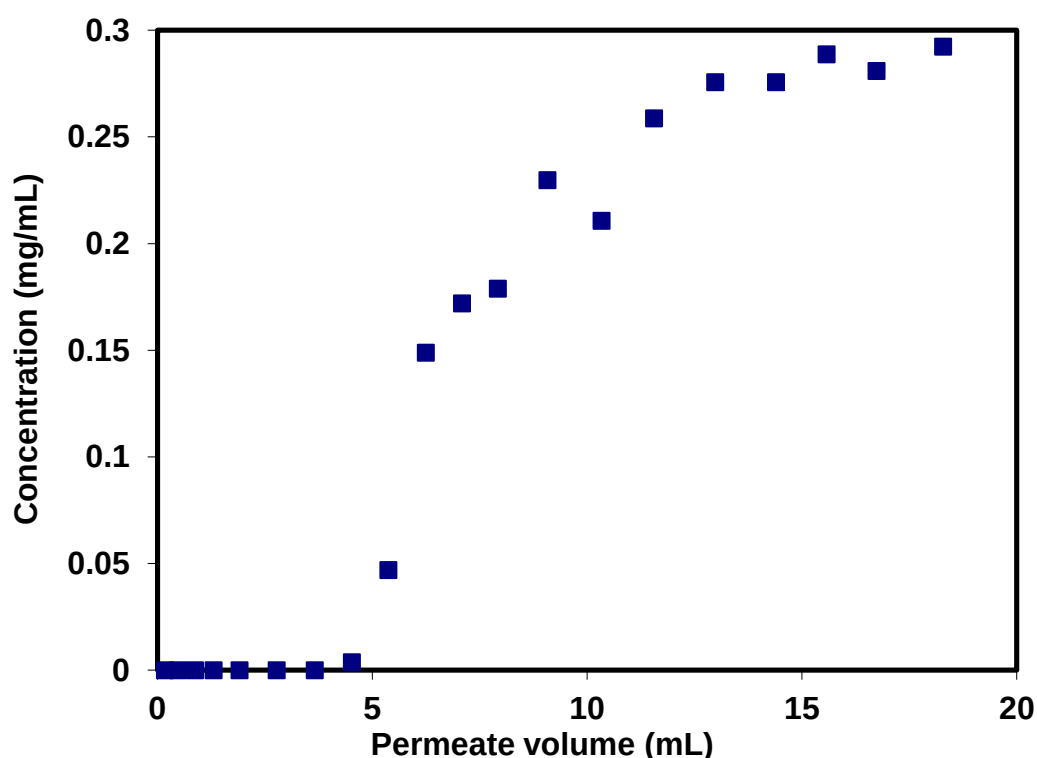


Figure 4.5. Breakthrough curve during passage of a 0.3 mg/mL Con A solution through a nylon membrane modified with poly(MES)-NTA-Cu²⁺. The Con A solution in 20 mM phosphate buffer (pH 6) was passed through the membrane at a flow rate of 1 mL/min. The membrane had an exposed area of 3.1 cm².

To examine glycoprotein capture by Con A immobilized via Cu²⁺ affinity, we determined the asialofetuin (a glycoprotein binding to Con A) binding capacity for nylon membranes containing poly(MES)-NTA-Cu²⁺, poly(MES)-NTA-Cu²⁺-Con A and poly(MES)-NTA-Cu²⁺-Con A/BSA (**Figure 4.6**) (40-42). For the membrane with poly(MES)-NTA-Cu²⁺-Con A, the asialofetuin binding capacity was 40 mg/cm³ of membrane. Unfortunately, asialofetuin binds to Cu²⁺ as well as to Con A, and the binding capacity of the membrane with only poly(MES)-NTA-Cu²⁺ (no Con A) was 55 mg Asialofetuin /cm³ of membrane. After trying to block excess Cu²⁺ sites and other

non-specific binding sites with BSA, the asialofetuin binding capacity decreased to about 8 mg/cm³ of membrane. The low capacity might stem from steric hindrance to binding sites. The molecular mass of asialofetuin is 48.4 kDa, whereas that of Con A is 104 kDa. If each of the four subunits of Con A captured one asialofetuin molecule, the binding capacity would be about 120 mg asialofetuin/cm³ of membrane based on the immobilization of 69 mg Con A/cm³ of membrane.

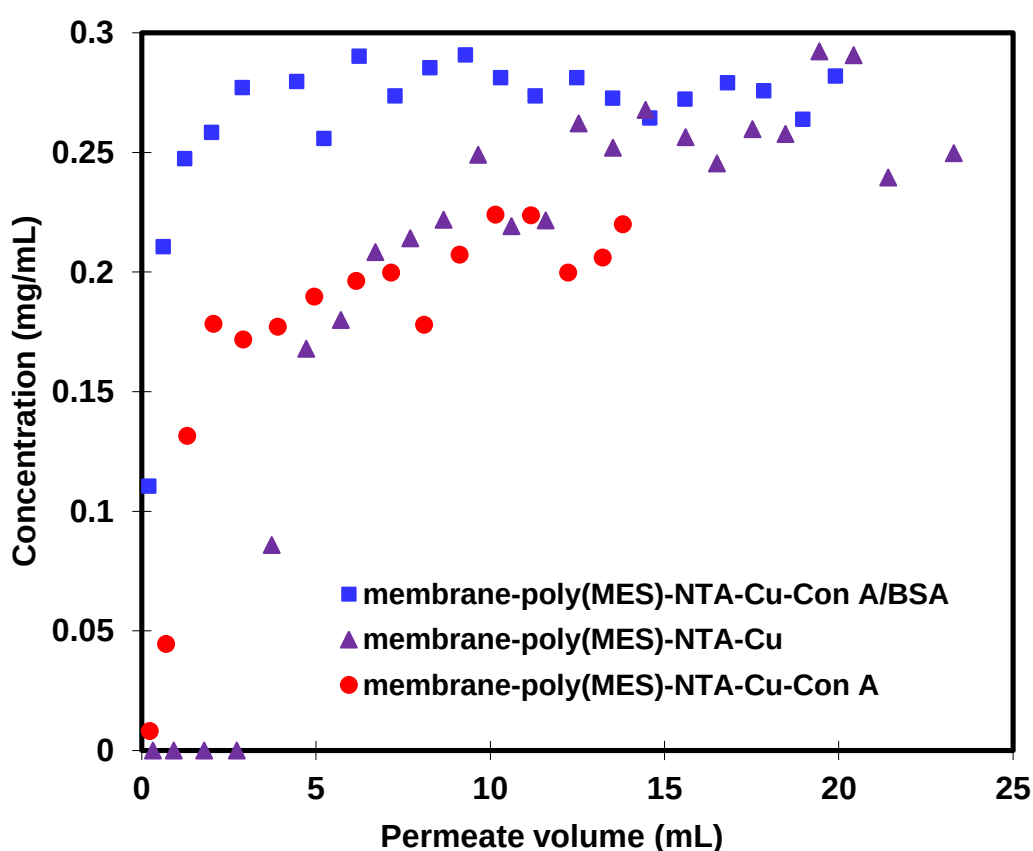


Figure 4.6. Breakthrough curves during the passage of 0.3 mg/mL asialofetuin through membranes modified with membranes with poly(MES)-NTA-Cu²⁺, poly(MES)-NTA-Cu²⁺-Con A or poly(MES)-NTA-Cu²⁺-Con A/BSA. Asialofetuin was dissolved in pH 7.4 Tris buffer and passed through the membrane (3.1 cm²) at 1 mL/min.

4.3.3 Con A immobilization via covalent interactions and subsequent glycoprotein binding

We also immobilized Con A via reaction of some of its lysine residues with the activated –COOH groups of poly(MES). In this anchoring strategy, the pH of the buffer plays an important role. Normally, the rate of amide formation would increase at high pH due to deprotonation of amino groups. However, Con A aggregates at pH values > 7 (43, 44). Thus we attempted to immobilize Con A at pH 6 using a relatively long reaction time of 4 h. This strategy immobilizes only 10 mg of Con A/cm³, as determined from decrease in the concentration of Con A in the immobilization solution after immobilization. Considering Con A's dimensions of 4.2 × 4 × 3.9 nm, the binding capacity indicates a single layer protein binding (45). Moreover, the Asialofetuin binding capacity is only 5 mg/cm³ of membrane (Figure 4.7). This Asialofetuin binding capacity suggests that only 25% of the binding sites on Con A are accessible, although this percentage should be viewed with caution due to uncertainties in determining low binding capacities. The random covalent linkages may disrupt the protein structure or limit access to the binding site (46).

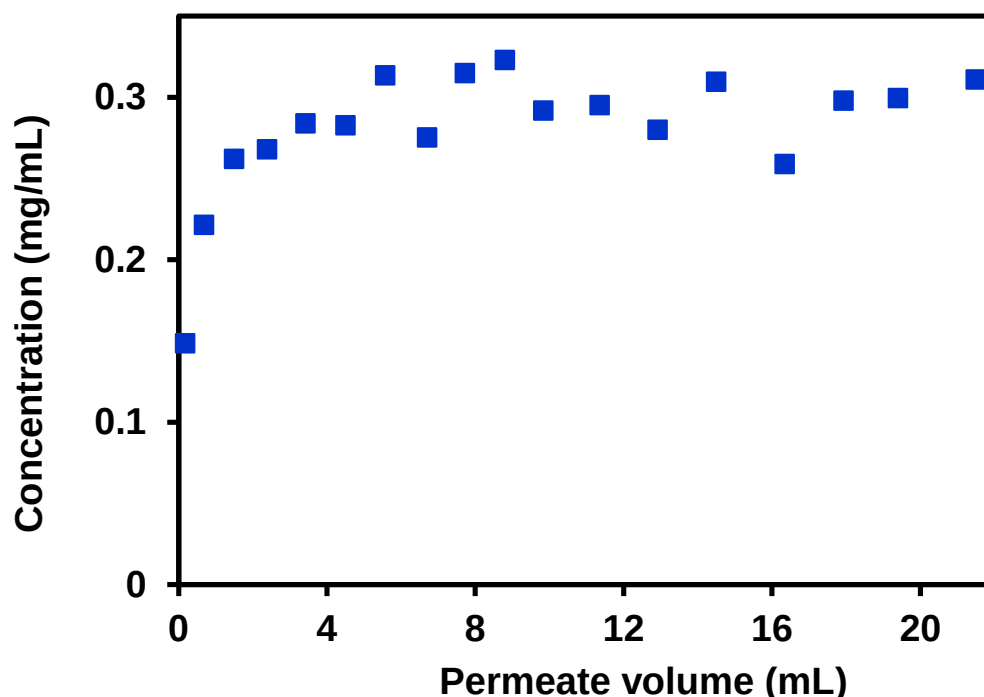


Figure 4.7. Breakthrough curve of asialofetuin for membranes with covalently immobilized Con A. Asialofetuin was dissolved in pH 7.4 Tris buffer at a concentration of 0.3 mg/mL, and this solution was passed through the membrane at 1 mL/min. The membrane area was 3.1 cm².

In an effort to protect the binding site, we modified Con A with methyl- α -D-mannopyranoside during immobilization. Binding of this sugar to Con A should prevent formation of covalent bonds to the binding site (33). The presence of methyl- α -D-mannopyranoside does not decrease the amount of Con A immobilization. However, after removal of methyl- α -D-mannopyranoside, the asialofetuin binding capacity was the same as that for Con A immobilized without methyl- α -D-mannopyranoside.

All the experiments were repeated using PAA/PEI/PAA-modified membranes with similar methods to immobilize Con A via Cu²⁺ affinity or covalent linkages. However,

the binding capacity for glycoprotein was again not extensive.

4.4. Conclusion

Using macroinitiator adsorption, a 5-min polymerization of MES within membrane pores successfully created a platform that binds at least 100 mg of lysozyme/cm³ of membrane and still maintains sufficient hydraulic permeability for flow with a peristaltic pump. After functionalization with NTA-Cu²⁺ complexes, these membranes can capture 69 mg Con A/cm³. However, after blocking nonspecific binding sites with BSA, this high Con A binding capacity gave an asialofetuin binding capacity of only 10 mg/cm³ of membrane. Even when Con A was immobilized with its binding sites protected, asialofetuin binding was not extensive. There are two possible reasons for the low glycoprotein capture: 1) The immobilization process might damage Con A, which is a pH-sensitive protein (43), or more likely 2) The immobilization of Con A leads to significant steric hindrance to binding.

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Chapter 5. Rapid and Controlled Protein Digestion in Porous Membrane Reactors Containing Covalently Immobilized Trypsin

The previous chapters described the development of a membrane platform for purification of proteins, including His-tagged protein and glycoproteins. Convective mass transport and minimal radial diffusion distances allow rapid protein purification, and modification of membrane micropores with PEMs often gives a high protein binding capacity. Thin (~100 μm) microporous membranes also provide a unique platform for creating enzyme reactors. This chapter describes covalent immobilization of trypsin in a PAA-modified membrane and examines the enzymatic activity of the immobilized enzyme. The thin membrane reactor affords fine control over digestion time to control peptide lengths and enhance protein analysis with MS.

5.1. Introduction

Since the sequencing of the human genome, research interests have shifted to uncovering the secrets of the human proteome (1, 2). An understanding of protein structures, PTMs and protein-protein interactions sometimes provides valuable information about the development of diseases such as cancer (3). MS is the most comprehensive and versatile analytical technique to identify proteins and their PTMs (4-6), and driven by the need for large-scale protein analysis, MS techniques continue evolving to give higher resolution, faster analysis, and higher mass accuracy (5-7). However, even with recent advances in MS, analysis of intact proteins with molecular weights greater than 50 kDa is challenging (8-10). As a result, proteolytic digestion to

convert proteins to peptides is a vital step in sample preparation for successful MS analysis (4, 11). Conventionally, digestion occurs after directly mixing the proteolytic enzyme with proteins solutions. Several proteases including chymotrypsin (12), trypsin (13), Lys-C (14) and Asp-N (15) are effective, but trypsin, which cleaves at the carboxyl side of Lys or Arg when neither is followed by Pro, is the most popular enzyme due to its high cleavage specificity and low cost (16). The enzyme to substrate ratio is usually low to avoid protease self-digestion, but this low ratio leads to long digestion times for complete digestion (17).

To enhance the protein-digestion rate, several research groups developed reactors containing immobilized trypsin (18-21). Substrates for trypsin immobilization include polymer plates (22), membranes (11, 23, 24), monoliths (25), and microfluidic channels (26, 27), and enzyme anchoring can occur via hydrophobic (23, 27), covalent (28) or electrostatic interactions (11, 24). Membranes present a unique platform for enzyme reactors because their small thickness causes low transmembrane pressure drops. As a result rapid flow rates through the membrane can yield digestion times as low as 1 msec. (11, 24). Xu and Tan from the Bruening group already developed trypsin and pepsin membrane reactors using electrostatic interactions to immobilize the enzyme (11, 24). Millisecond residence times in these membranes lead to large peptides that facilitate protein analysis. Unfortunately, at high ionic strengths, the electrostatic immobilization is insufficient to prevent enzyme leaching. Thus, this chapter focuses on immobilizing trypsin on the membrane via covalent bonds to enable digestion under harsh conditions.

Similar to previous chapters, adsorption of a layer of PAA in the membrane provides

free –COOH groups for extensive enzyme immobilization using NHS/EDC coupling. Importantly, PAA also serves as a shielding layer on the porous surface to minimize nonspecific binding and enhance the recovery of digested peptides (29, 30). This chapter describes enzyme immobilization, quantification of the enzymatic activity, and examination of controlled digestion of model proteins in the trypsin-containing membranes.

5.2. Experimental section

5.2.1. Materials

Hydroxylated nylon (LoProdyne® LP, Pall, 1.2 μm pore size, 110 μm thick, 50 % porosity) was cut into 2.5-cm disks. Trypsin from bovine pancreas (type I, 12200 units/mg solid), benzamidine hydrochloride, N_α -benzoyl-L-arginine ethyl ester hydrochloride (BAEE), poly(acrylic acid) (PAA, average molecular weight $\sim 100,000$, 35% aqueous solution), N -(3-dimethylaminopropyl)- N' -ethylcarbodiimide hydrochloride (EDC), N -hydroxysuccinimide (NHS), bovine β -casein and apomyoglobin (protein sequencing grade from horse skeletal muscle, salt-free lyophilized powder) were purchased from Sigma-Aldrich. Urea (J.T.Baker), Sodium phosphate (dibasic and monobasic) and Ammonium bicarbonate were obtained from Columbus Chemical. Acetonitrile (ACN) was obtained from Mallinckrodt Baker. Buffers were prepared using analytical grade chemicals and deionized (Milli-Q, 18.2 $\text{M}\Omega\text{ cm}$) water, and Sep-Pak® C18 Cartridges (100 mg, 55-92 μm beads, Part No.WAT023590) from Waters were employed for desalting.

5.2.2. Covalent immobilization of trypsin in membranes

Membrane modification employed an Amicon cell (Model 8010, 10 mL, Millipore) connected to a peristaltic pump. Hydroxylated nylon 6,6 membranes (2.5 cm diameter, 1.2 μ m pore size) were exposed to UV/ozone for 15 min, placed in the Amicon cell, and 5 mL of a solution containing 20 mM PAA (pH 3) was passed through the membrane using a peristaltic pump. Subsequently, 20 mL of deionized water was pumped through the membrane at 1 mL/min. The adsorbed PAA was activated by circulating an aqueous solution containing 20 mM NHS and 20 mM EDC through the membrane for 2 hours followed by passage of 20 mL of water and 20 mL of ethanol through the membrane. Subsequently, 5 mL of 0.4 mg/mL trypsin solution in 20 mM phosphate buffer (pH 7.6) supplemented with 20 mM benzamidine was circulated through the membrane for 2 hours to complete the covalent immobilization. These membranes were washed by passing 20 mL of phosphate buffer containing 20 mM benzamidine and 20 mL of 1 mM HCl through the membrane before drying with N₂ and storage at room temperature. In some cases, we examined membrane activity after 6 months of storage.

5.2.3. Quantitation of trypsin immobilization

The amount of trypsin bound to the membrane was determined by measuring the absorbance at 280 nm of the loading solution before and after circulating through the membrane. These studies employed a PerkinElmer Lambda 25 spectrophotometer and calibration solutions containing 20 mM benzamidine and trypsin at a concentration range of 0-0.3 mg/mL. Control experiments employed membranes with PAA but no NHS/EDC

activation and membranes without any modification. Because NHS absorbs at 280 nm under basic conditions, 2.5 mL of standard or sample solution was mixed with 0.5 mL of 1 M HCl before absorbance measurements (31).

5.2.4. Quantification of enzyme activity

We determined the proteolytic activity of trypsin both in solution and in membranes using the trypsin substrate BAEE dissolved in 10 mM NH_4HCO_3 at pH 7.6.

5.2.4.1. In-solution enzyme activity

Ten μL of 1 mg/mL trypsin in 2.7 mM HCl was added to 3 mL of 1 mM BAEE in 10 mM NH_4HCO_3 (pH 7.6) buffer and stirred with a pipet for ~ 5 s. The UV absorbance at 253 nm was monitored every 10 s with 1 mM BAEE as background until the absorbance plateaued.

5.2.4.2. In-membrane enzyme activity

Twenty mM BAEE was passed through the membrane (2-cm exposed diameter) at flow rates of 60 mL/hour, 40 mL/hour, 30 mL/hour, 20 mL/hour, 15 mL/hour, 10 mL/hour, 8 mL/hour and 6 mL/hour using a 10-mL syringe pump. With a membrane thickness of 110 μm and 50% porosity (values provided by Pall), these flow rates give residence time of 0.017 to 0.17 min. Because BAEE has a very strong absorbance at 253 nm, all the samples were diluted 20-fold with phosphate buffer before measurement against a 1 mM BAEE background.

5.2.5. Protein digestion

Proteins were dissolved in water at 10 mg/mL, separated into 100 μ L aliquots, dried using a SpeedVac and stored at -20 °C until use.

5.2.5.1. In-solution digestion

For in-solution digestion without denaturation, 10 μ L of freshly made 0.5 mg/mL trypsin solution in 1 mM HCl was added to 0.1 mg/ml protein solutions in 10 mM NH_4HCO_3 to achieve a 1:20 ratio of trypsin to substrate protein. After the desired digestion time, the reaction was quenched by addition of 11 μ L acetic acid.

5.2.5.2. Membrane digestion

For protein digestion, the membrane was cut to fit into a miniature membrane holder (the holder exposes 0.02 cm² of external membrane area, which was connected to a syringe pump (KDS210, KD scientific) as shown in **Figure 5.1** (11). Before digestion, pH 7.6 buffer was passed through the membrane for 30 min to activate the immobilized trypsin. (Prior to storage the membrane had been rinsed with 1 mM HCl). For digestion at different pH values, the membrane was activated with phosphate buffers adjusted to the pH of the digestion. Solutions containing 0.1 mg/mL protein in 10 mM NH_4HCO_3 buffer were sequentially passed through the trypsin containing membrane at flow rates of 0.12 mL/hour, 1.2 mL/hour, 12 mL/hour and 120 mL/hour. The corresponding residence times are 3.3 s, 0.33 s, 33 ms and 3.3 ms. For membrane digestions without salts and urea, 50 μ L of permeate was collected and dried with a Speed-Vac, and then reconstituted

in MS buffer (50 μ L of 1% acetic acid/49% H₂O/50% methanol). For digests with urea, desalting was performed using a C18 Sep-Pak cartridge.

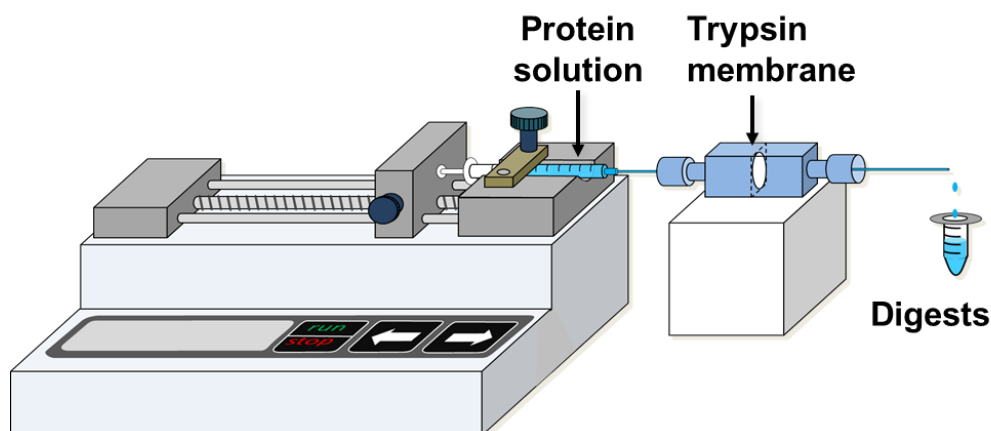


Figure 5.1. Apparatus for in-membrane protein digestion. A syringe pump forces a protein solution through a miniature trypsin-containing membrane. Drawn by Zhefei Yang.

5.2.6. Desalting using a C18 Sep-Pak cartridge

Digests containing urea were acidified with formic acid (FA) to get a 0.5% final FA concentration and desalted using a C18 cartridge (syringe cartridge with 1 mL volume). The C18 cartridges were first washed and equilibrated with acidic solutions by sequentially passing 3 mL of 0.05% FA in 99.95% ACN and 3 mL of aqueous 0.05% FA through the cartridge. 150 μ L of acidified protein solution was then forced through the cartridge followed by 0.5 mL of 0.05% FA for washing. Protein bound to the solid phase was recovered by sequentially passing 0.2 mL of 0.05% FA buffers containing 40%, 60%, 80% and 99.95% ACN through the cartridge. All the above procedures were performed manually with a $\sim$ 0.7 mL/min flow rate. The combined elution fractions were finally dried with a SpeedVac and reconstituted in MS buffer before analysis.

5.2.7. Mass spectrometry and data analysis

All the digests were analyzed with ESI-MS. Forty μL of reconstituted protein digest in MS buffer was loaded into a Whatman multichem 96-well plate and sealed with Teflon Ultrathin Sealing Tape. The samples were introduced into the high-resolution accurate mass Thermo LTQ Orbitrap Velos mass spectrometer using an Advion Triversa Nanomate nanoelectrospray ionization (nESI) source. The spray voltage was 1.4 kV, and the gas pressure was 1.0 psi. High-resolution mass spectra were acquired in positive ionization mode using the FT analyzer operating at 100,000 resolving power with relative intensity as the Y-axis. Peptides were identified manually by comparing the experimental data to the theoretical peptides generated from the ProteinProspector MS-Product program (32). For the theoretical peptide generation, the data base was user protein, the enzyme was set to trypsin, the maximum missed cleavage was 99, the peptide mass was 200 to 30000 Da, the minimum peptide length was 2 amino acids and the digestion was performed with the protein sequence without the signal peptides.

5.3 Results and discussion

Membranes are attractive solid supports for enzyme reactors because they allow fine control over residence time through variation of flow rate. The first two subsections below describe trypsin immobilization in the membrane and studies of the immobilized enzyme's activity. Subsequent subsections discuss in-membrane protein digestion and membrane stability.

5.3.1. Covalent immobilization of trypsin in membranes

Figure 5.2 shows our strategy for enzyme immobilization, which includes adsorption of PAA, activation of $-\text{COOH}$ groups with NHS/EDC, and covalent coupling via amide linkages (33). Several previous studies reported covalent immobilization using accessible primary amine groups on trypsin (25, 28, 34-37), mainly through reactions with epoxy or aldehyde groups on the solid support (38). Amide coupling using NHS/EDC is more rapid than reaction of amine groups with epoxides or aldehydes, and the amide bond is more stable than the imine formed by reaction with aldehydes. At pH 7.6, NHS esters that do not react with amine groups undergo hydrolysis to reform $-\text{COOH}$ groups and avoid possible covalent capture of other proteins during digestion with trypsin-containing membranes. To avoid trypsin self-digestion, we add benzamidine as a competitive inhibitor during the immobilization step (34, 35). The benzamidine may also minimize the formation of covalent bonds with amino acids near the active site of trypsin and stabilize the protein tertiary structure (35, 39).

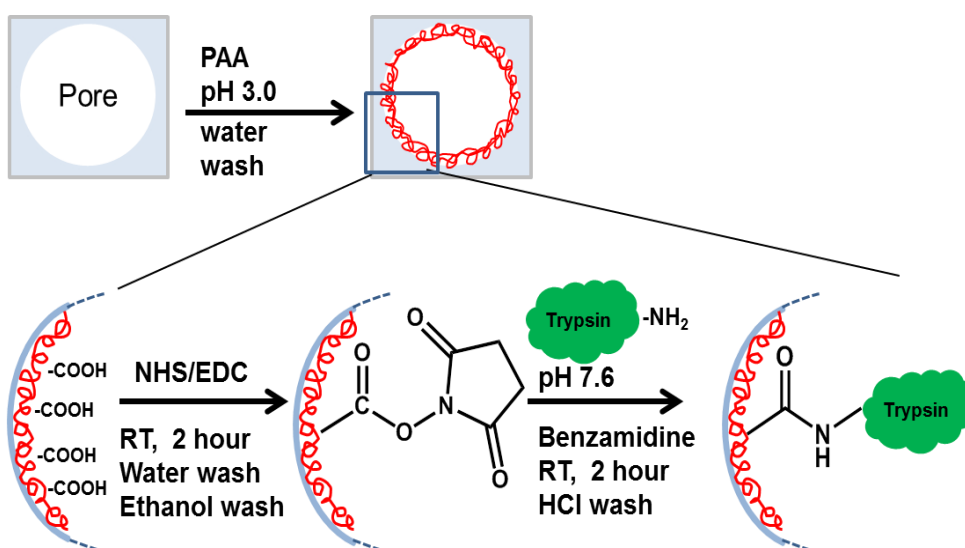


Figure 5.2. Covalent immobilization of trypsin to PAA adsorbed in a membrane pore.

Based on the decrease in the concentration of trypsin in the solution circulated through the membrane, the modified membrane contains ~ 35 mg of trypsin per cm^3 of membrane or ~ 70 mg trypsin per cm^3 of pores, assuming 50% porosity. This binding capacity is higher than the 11 mg per cm^3 of membrane that we achieved previously through adsorption of poly(styrene sulfonate) in the membrane and subsequent electrostatic capture of trypsin (11, 24). Moreover, the trypsin “concentration” in membrane pores is 2800 times the $0.025 \text{ mg}/\text{cm}^3$ trypsin concentration in a typical in-solution digestion (11), and this high concentration enables rapid digestion. Control experiments on bare nylon membranes showed <5 mg trypsin per cm^3 of membrane.

Hydrolysis of active esters during trypsin binding could lead to some immobilization of trypsin through electrostatic interactions with $-\text{COO}^-$ groups. However, washing of trypsin-modified membranes with buffers containing as much as 1 M NaCl, led to no detectable absorbance at 280 nm, indicating minimal protein elution under conditions that should disrupt electrostatic interactions. In contrast, electrostatic binding of trypsin to PAA adsorbed in nylon membranes (no NHS/EDC activation) captured ~ 40 mg trypsin per cm^3 of membrane, but elution with 1 M NaCl removed all the bound trypsin. These data imply that trypsin capture after NHS/EDC activation occurs through covalent bonds.

5.3.2. Proteolytic activity of Trypsin Membranes

After creating trypsin-modified membranes, we initially examined their enzyme activity spectrophotometrically by monitoring the cleavage of the ester group of BAEE, a

synthetic trypsin substrate (28, 35, 36, 40). The UV absorbance at 253 nm due to the digestion product, *N*_a-benzoyl-L-arginine (BA), is proportional to the rate of ester cleavage (41). Trypsin activity typically follows Michaelis-Menten kinetics, where equation (5.1) describes

$$V = V_{max} \frac{[S]}{[S] + K_M} \quad (5.1)$$

the reaction rate, *V* or more explicitly $-\frac{d[S]}{dt}$, in terms of the rate when the enzyme is saturated with substrate, *V*_{max}, the substrate concentration, [S], and the Michaelis constant, *K*_m. Usually, a plot of $\frac{1}{V}$ versus $\frac{1}{[S]}$ yields values for *V*_{max} and *K*_m. However, for the membrane reactor, the value of [S] varies significantly from the feed to the permeate side of the membrane. At low BAEE feed concentrations, the high trypsin loading in the pores leads to essentially complete BAEE cleavage. Thus, we employ equation (5.2), the integrated Michaelis-Menten equation, to determine kinetic parameters. In this expression

$$t = \frac{K_M}{V_{max}} \ln \left(\frac{[S]_0}{[S]} \right) + \frac{[S]_0 - [S]}{V_{max}} \quad (5.2)$$

t is the digestion time and [S]₀ is the initial substrate concentration. Specifically for the case where the membrane behaves as a plug-flow reactor, *t* is the residence time, *t*_{res}, of solution in the membrane, [S]₀ is the substrate concentration in the feed, and [S] is the substrate concentration in the membrane permeate. We employ equation (5.3) to calculate the residence time, assuming a porosity, ε, of 0.5; a membrane thickness, *l*, of 0.011 cm; and a membrane area, *A*, of 3.1 cm².

$$t_{res} = \frac{Al}{Q\varepsilon} \quad (5.3)$$

Variation of the volumetric flow rate, *Q*, using a syringe pump affords different

residence times, and **Figure 5.3** shows a plot of the BAEE concentration in the membrane permeate as a function of the residence time. Fitting of the data in **Figure 5.3** with equation (5.2) yields $V_{max}=1$ mM/s and $K_m \cong 4$ mM. However, given that the data in **Figure 5.3** are essentially linear, the fit does not depend significantly on K_m , so that value is highly uncertain.

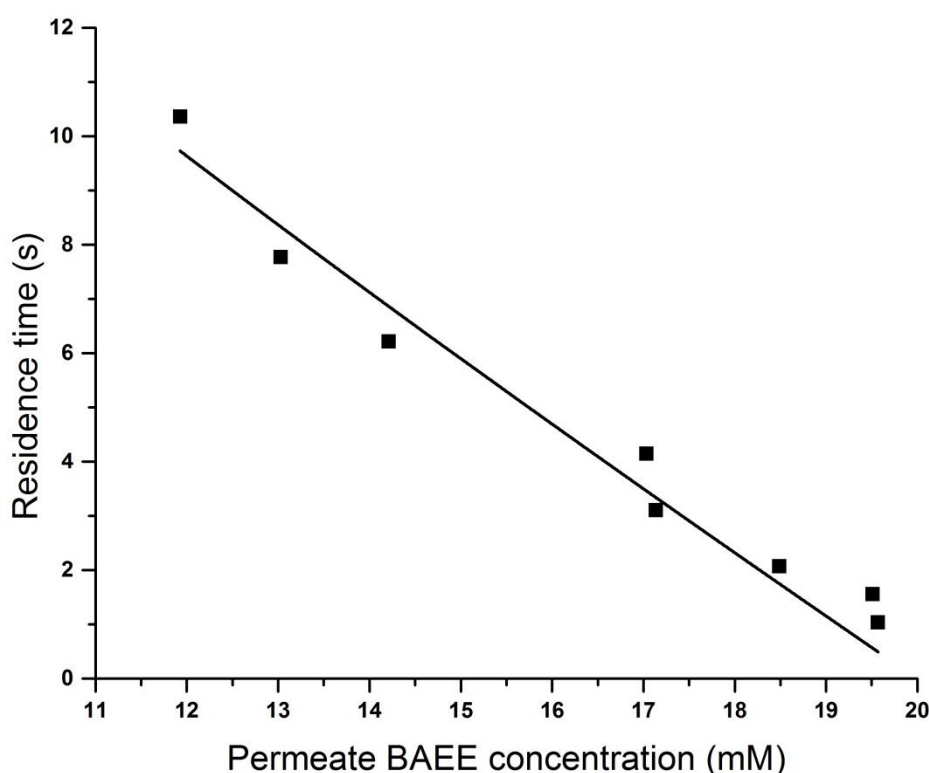


Figure 5.3. Residence time as a function of the BAEE concentration in the permeate for BAEE digestion during passage through a trypsin-modified membrane. The feed solution contained 20 mM BAEE. The curve is the fit to the data using equation (5.2).

We also determined kinetic parameters using in-solution digestion and with the same fitting method. In this procedure, we collected the UV absorbance every 10 s until the reading plateaued. From the fitting shown in **figure 5.4**, we get $V_{max} = 0.013$ mM/s and $K_m = 0.35$ mM in a typical solution digestion, and the V_{max} is consistent with reported

values of $V_{max} = 0.0134$ mM/s determined using equation (5.1) (35). However, the V_{max} value in the membrane is about 75 times that in solution because of the high trypsin “concentration” in the membrane pores. Nevertheless, immobilization does decrease the enzyme activity, as the enzyme concentration in the membrane pores is 2800 times that in solution.

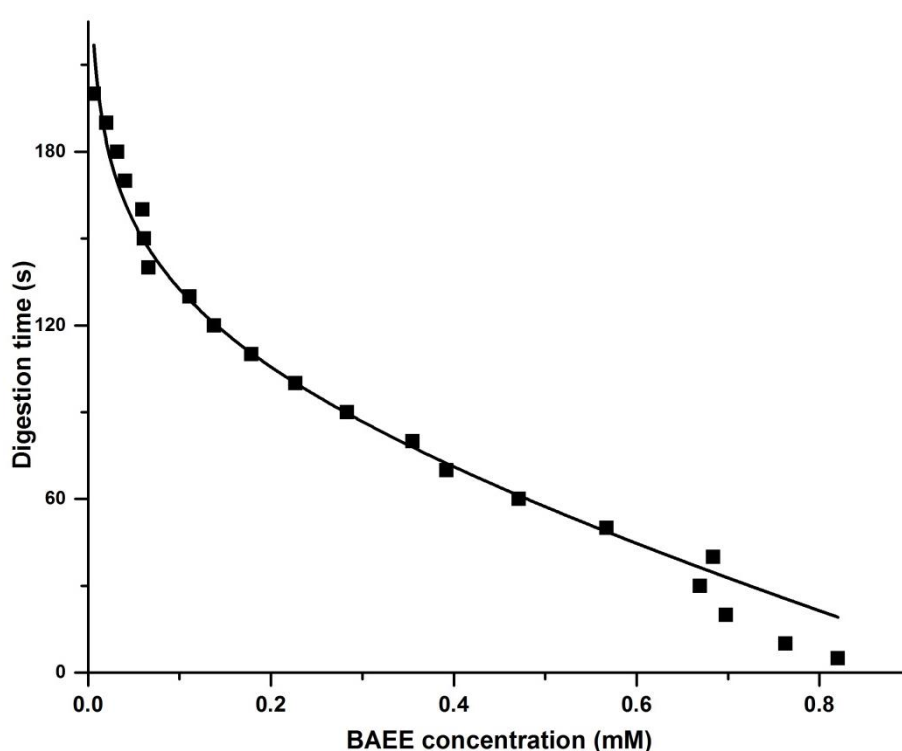


Figure 5.4. In-solution digestion time as a function of the evolving BAEE concentration. The initial BAEE concentration in the solution was 1 mM. The curve is the fit to the data using equation 5.2.

5.3.3. Controlled β -casein digestion as a function of residence time

In addition to rapid digestion, protease-containing membranes provide a platform for controlling the extent of protein cleavage by varying residence times down to the ms

level. β -casein served as our initial model protein for examining the extent of protein digestion as a function of flow rate because digestion can occur in 10 mM NH_4HCO_3 without denaturation. The electrophoretic gel in **Figure 5.5** shows the molecular weights of digestion products obtained at flow rates of 120 mL/hour, 12 mL/hour and 0.12 mL/hour, which correspond to residence times of 3.3 ms, 33 ms and 3.3 s, respectively. The gel shows a clear trend toward increased protein digestion at longer residence times. The 3.3-ms residence times leads to a significant amount of intact protein along with several proteolytic peptides with masses between 10 and 25 kDa, whereas a 3.3-s residence times yields no visible peptides, indicating complete digestion into peptides with masses <10 kDa. The intermediate residence time of 33 ms gives minimal intact protein along with peptides with masses between 10 and 25 kDa.

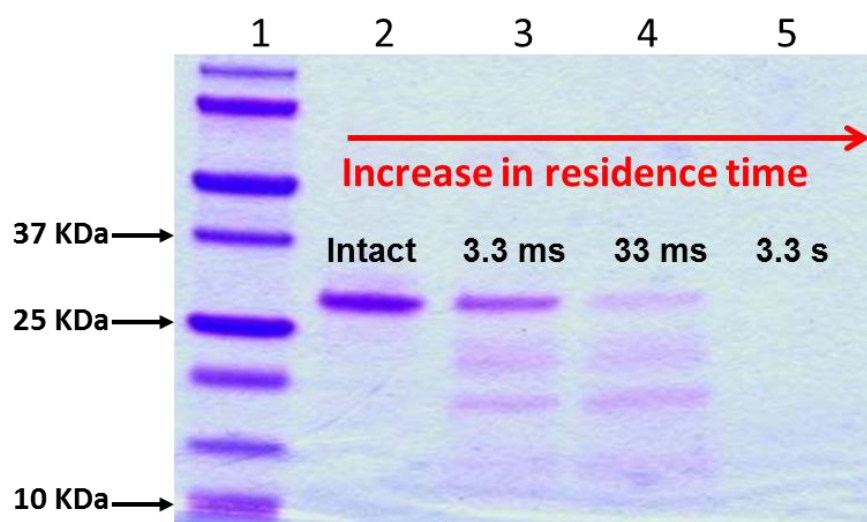


Figure 5.5. SDS-PAGE of intact β -casein (lane 2) and membrane digests of β -casein obtained with different residence times (lanes 3-5). Five- μg protein samples were loaded for each lane, and lane 1 shows a protein ladder. Digestion occurred using 0.1 mg/mL β -casein in 10 mM NH_4HCO_3 .

We analyzed the digests obtained at different residence times with ESI-Orbitrap mass spectrometry using direct injection. **Figure 5.6** compares the deconvoluted mass spectra for digestion with 33-ms and 3.3-s residence times. For the 33-ms digestion (**Figure 5.6a**), as few as two peptides (e.g. 14 and 17) can cover the entire 209 amino acid sequence. In contrast, at the long residence time (3.3 s, **Figure 5.6b**), all detected peptides have masses less than 8 kDa, which is consistent with the gel electrophoresis (**Figure 5.5, lane 5**). The long residence times also lead to relatively high intensities of peptides with masses less than 1000. More missed cleavage occurs at short residence times to decrease the intensities of small peptides, e.g., with the 33-ms residence time, the signal for peptide 8 (amino acid (AA) 170-209) largely replaces signals for peptides 1 (AA 170-176), 2 (AA 177-183), and 4 (AA 184-209). Sometimes, researchers employ highly selective enzymes to obtain large peptides to achieve high sequence coverage (42, 43), but the peptide size depends on the protein sequence. The trypsin enzyme reactor may enable production of large peptides independent of sequence.

In addition to improving sequence coverage, controlled limited digestion may facilitate simultaneous analysis of multiple post translational modifications in single peptides (“combinatorial” PTM characterization) (44). Such studies can show whether PTMs occur simultaneously or randomly. β -casein is phosphorylated at Ser15, Ser17, Ser18, Ser19 and Ser35. Previous analysis of β -casein phosphorylation based on small tryptic phosphopeptides inevitably required phosphopeptide enrichment from the complete β -casein digest (45). The existence of one Arg and three Lys residues between Ser35 and the other four phosphorylation sites makes the simultaneous characterization

of the five phosphorylation sites on a single peptide almost impossible after traditional in-solution tryptic digestion. However, peptide 17 (**Figure 5.6a**, AA 1-107) contains all five phosphorylation sites on β -casein. The signal of this peptide is likely low in positive-ion mode because of the 5 acidic phosphate groups, but it clearly shows that all five phosphorylation events occur together on this peptide.

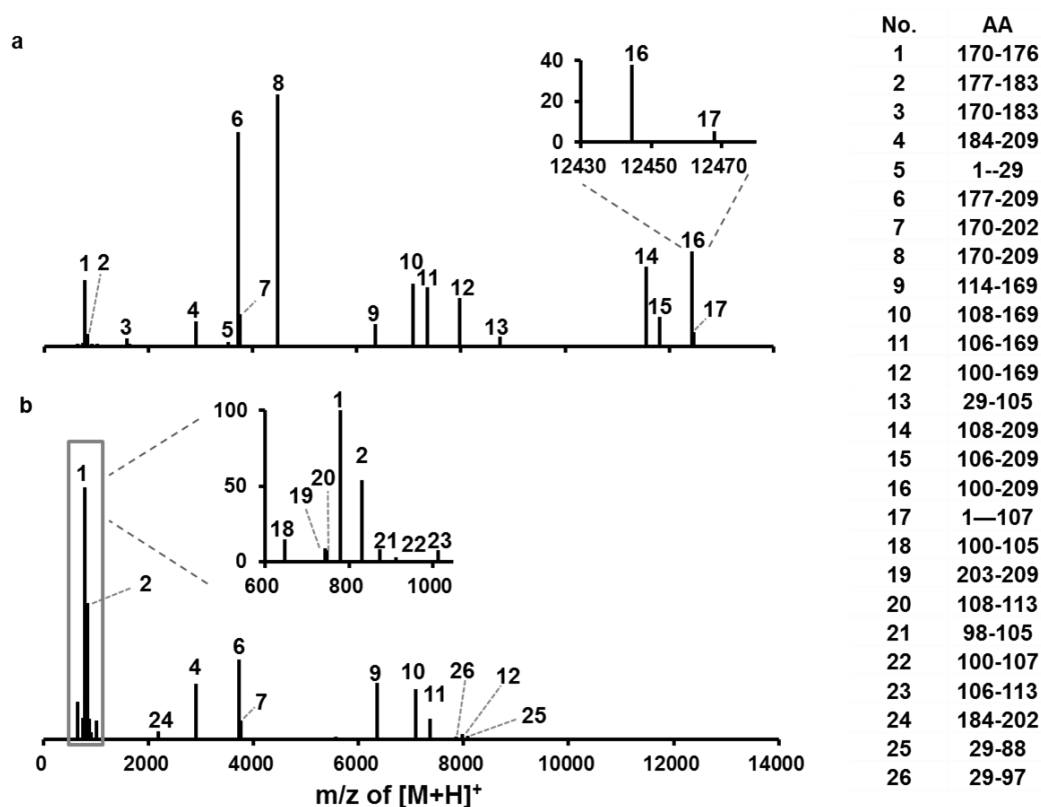


Figure 5.6. Manually deconvoluted ESI-Orbitrap mass spectra of 0.1 mg/mL β -casein digested with residence times of (a) 33 ms and (b) 3.3 s in a trypsin-modified membrane. Signals above 1% of the highest signal were assigned to specific peptides by comparison to theoretical m/z values. The normalized intensities are the sum of signal intensities for all detected charge states of a given peptide, and the spectra show the peptides only at the +1 charge state for the monoisotopic mass.

5.3.4. Apomyoglobin digestion with high concentrations of urea and variable pH

After successful β -casein digestion under native conditions (10 mM NH_4CO_3), apomyoglobin served as a model protein for membrane digestion under denaturing conditions because its tryptic map is well documented (35). Apomyoglobin is a monomeric protein with 153 amino acid residues and no disulfide bonds, but it undergoes slow tryptic proteolysis at pH 7-8 because of its compact hydrophobic core at neutral pH (11, 46, 47). **Figure 7a** shows the ESI-Orbitrap mass spectrum of apomyoglobin after in-solution digestion at pH 7.6 for 30 s. The appearance of dominant signals from intact protein (marked with *) confirms the slow proteolysis under this condition. Apomyoglobin digestion in the trypsin-containing membrane (pH 7.6 without urea) for 3.3 s (**Figure 7b**) provides 100% sequence coverage from proteolytic peptides, and the intact protein peaks are less intense than for the 30-s in-solution digestion. Consistent with the Michaelis-Menten study in section 4.3.2, this confirms that the membrane reactor has a faster digestion rate. After addition of 6 M urea to the apomyoglobin solution, a 3.3-s membrane digestion shows 100% sequence coverage and no detected intact protein peaks in the spectrum (**Figure 7c**), indicating that the membrane reactor still works in the presence of a high concentration of urea. This is especially important for protein samples with low solubility. In-solution digestion in 6 M urea is typically not possible due to loss of enzymatic activity (48).

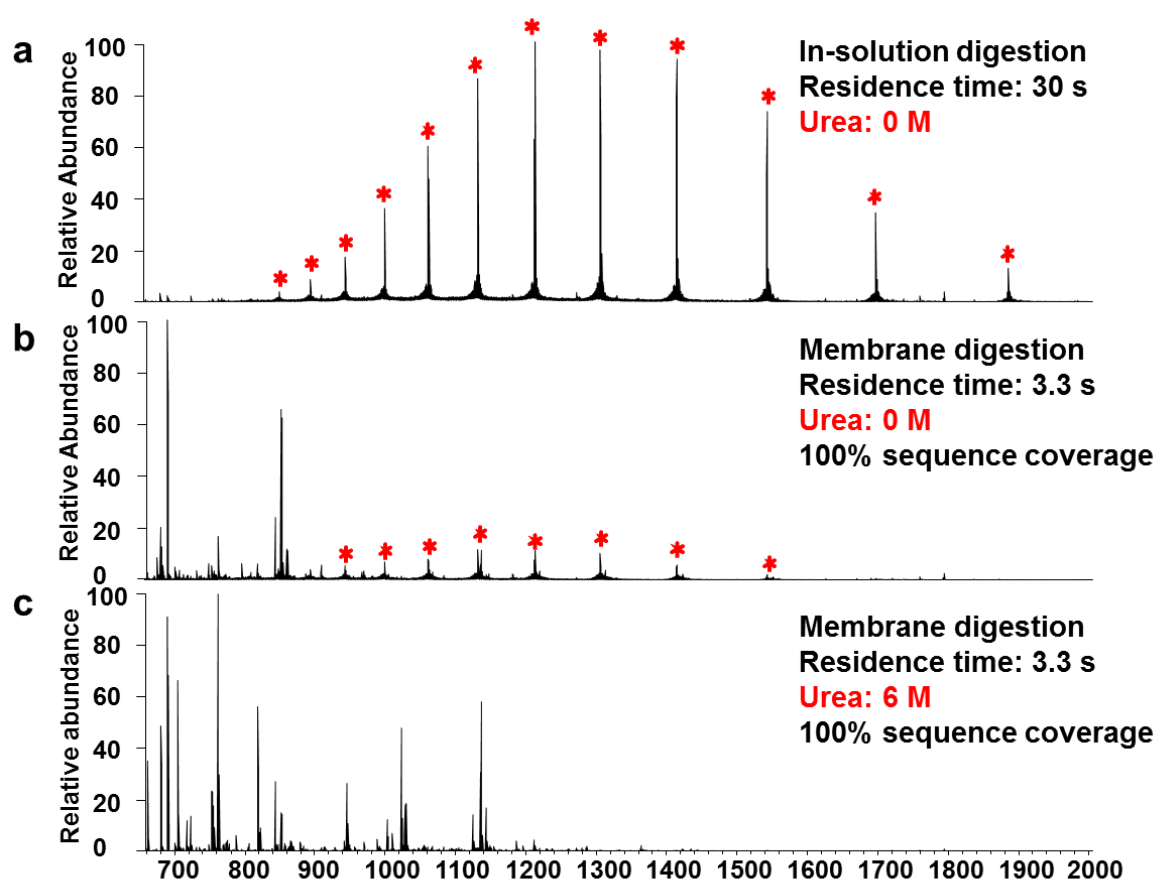


Figure 5.7. ESI-Orbitrap mass spectra of 0.1 mg/mL apomyoglobin digested (a) in-solution in 10 mM, pH 7.6 NH_4HCO_3 for 30 s; (b) in a trypsin-modified membrane for 3.3 s using 10 mM, NH_4HCO_3 , pH 7.6, no urea; and (c) in a trypsin-modified membrane for 3.3 s using 10 mM, NH_4HCO_3 , pH 7.6 with 6 M urea. For (c), urea was removed using a C18 Sep-Pak cartridge (peaks labeled with * correspond to intact apomyoglobin).

The extent of apomyoglobin digestion also depends on pH (46, 47, 49). Our previous studies using membranes with electrostatically immobilized pepsin showed that digestion of apomyoglobin at low pH occurs in residence times as short as 0.02 s (11). This study explores the effect of pH on apomyoglobin digestion in membranes with covalently immobilized trypsin. **Figures 8a and 8b** show mass spectra of apomyoglobin

digests performed in solution and in trypsin-containing membranes, respectively, at pH 5. No urea was added to these solutions. With membrane digestion at pH 5, no intact protein signals appear, which indicates a more complete digestion compared to proteolysis at pH 7.6 (compare **Figures 8b** and **7b**). Presumably, partial apomyoglobin denaturation at pH 5 enhances digestion (45, 46, 48). Previous studies suggest that trypsin is inactive at pH below 6 (37), but these results imply an extended working pH range from 5 to 8.5 for trypsin immobilized in membranes. The relatively wide working range with the trypsin-containing membrane will allow digestion under weakly acidic conditions that may denature many proteins. Although digestion at even lower pH can occur with pepsin (11), trypsin is attractive because of its higher specificity. For all the protein-digestion experiments, we observed no trypsin self-digestion peaks under all digestion conditions, which implies that the covalently immobilization gives a stable membrane reactor.

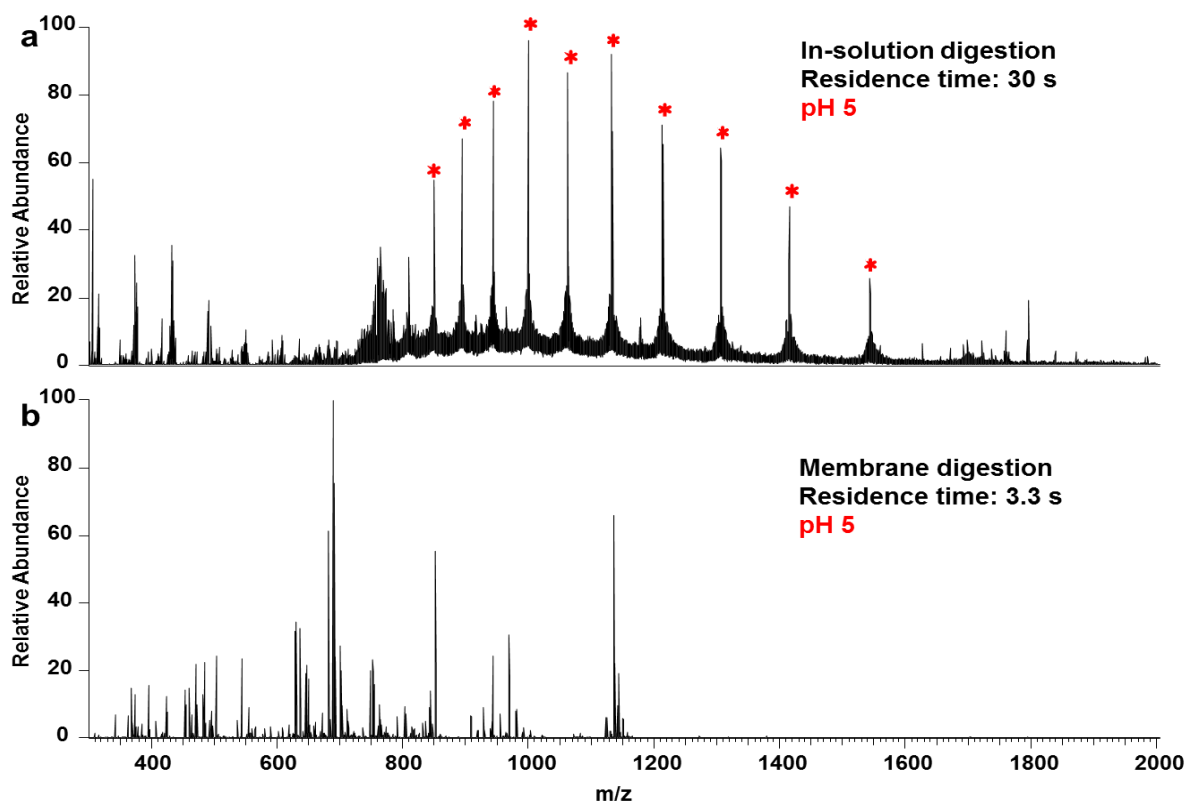


Figure 5.8. ESI-Orbitrap mass spectra of 0.1 mg/mL apomyoglobin digested (a) in solution for 30 s at pH 5 and (b) in a trypsin-modified membrane for 3.3 s at pH 5. Apomyoglobin was dissolved in 10 mM NH_4HCO_3 (peaks labeled with * correspond to intact apomyoglobin).

5.3.5. Stability of the trypsin membrane reactor

In addition to their rapid and controlled protein digestion, membrane reactors are also durable. Even with storage in water at room temperature, the membrane does not lose enzymatic activity after more than 24 hours when we examine BAEE digestion every hour in two time intervals (**Figure 5.9**). In contrast, in solution (pH 7.5) the enzyme completely loses its activity after this period of time (28). The hydrophilic PAA surface may reduce the denaturation of trypsin after immobilization (22, 28, 50). Typically, after fabrication, we dry the membrane and store it in a dessicator at room

temperature. After storage for at least 6 months, the membrane provides the same β -casein digestion results as shown in **Figure 5.6**.

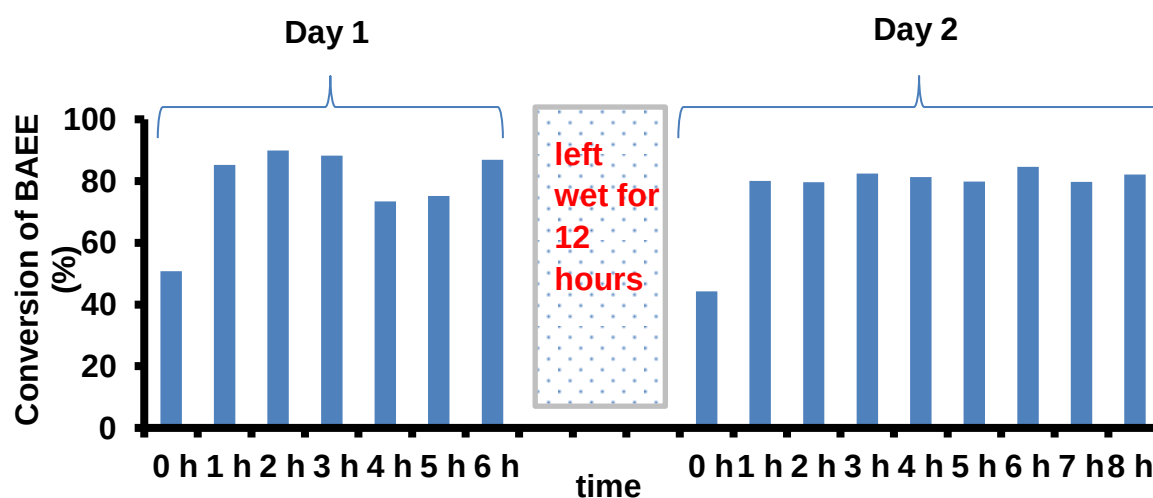


Figure 5.9. Conversion of BAEE in a continuous flow mode using a membrane with an exposed 2-cm diameter. The low conversion in the first tests on both days is due to sample dilution by the system dead volume. The feed solution contains 20 mM BAEE in 10 mM NH_4HCO_3 , and the flow rate was 10 mL/hour. h: hour.

5.4. Conclusion

Porous membranes modified with one layer of PAA bind 35 mg of trypsin/cm³ of membrane, and this high enzyme concentration leads to protein digestion that is 75 times faster than conventional in-solution digestion. In addition, the covalent immobilization stabilizes the enzyme and prevents leaching. The trypsin membrane reactor digests protein even in the presence of 6 M urea or at pH 5, which enables denaturation of proteins that typically resist digestion. When dry, membranes stored for over 6 months at room temperature are still active. A miniature membrane holder and syringe pump afford control over digestion times and, hence, peptide lengths. For β -casein, we found a large peptide (AA 1-107) that carries all the phosphorylation sites.

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Chapter 6. Summary and future work

6.1. Research summary

This dissertation describes my efforts to develop functionalized membrane platforms for protein purification and protein digestion prior to MS characterization. The functionalization starts with membrane modification using polymeric thin films. Chapter 2 describes my fundamental study of PEMs and shows that the properties of (PAH/PAA)_n films depend on deposition conditions. For films grown at pH 3, deprotonation of –COOH groups at neutral pH leads to a 500% increase in thickness upon swelling in water and creates binding sites throughout the film structure to capture multilayers of lysozyme through ion-exchange. Consistent with the ion-exchange mechanism, lysozyme binding to these PEMs depends on the pH and ionic strength of the binding buffers, and elution solution occurs in 1 M KSCN. (PAH/PAA)_n films adsorbed on Au-coated wafers at pH 3 bind large amounts of lysozyme at neutral pH without increasing surface roughness, presumably because lysozyme replaces water in the film. However, in the absence of lysozyme (PAA/PAH)_n films are not stable in a neutral pH buffer. Fortunately, film stability is greater in nylon membranes than on Au-coated wafers, which allows the development of stable, functional membranes.

Chapter 3 describes the development of Ni²⁺-containing membranes for isolation of His-tagged proteins. When deposited from pH 3 solutions, PAA/PEI/PAA films have a high density of free –COO[–] groups which are available for further modification with aminobutyl NTA to chelate Cu²⁺ or Ni²⁺ ions. Spongy nylon membranes modified with

PAA/PEI/PAA-NTA-Ni²⁺ enable rapid isolation of His-tagged CSN8 from cell extracts with > 95% purity and recovery within 30 min. With a commercial membrane holder, the whole process only requires 5 min.

Membranes with PAA/PEI/PAA-NTA-Ni²⁺ films successfully capture His-tagged proteins, but derivatization with aminobutyl NTA is inefficient and expensive. Additionally, weak metal-ion binding to unreacted –COO[–] groups of PAA leads to high metal-ion leaching. A newly designed polymer, PNTA, contains NTA groups and no isolated –COO[–] groups. Direct adsorption of PNTA simplifies formation of membranes with chelating groups, and importantly these modified membranes show less Cu²⁺ leaching than membranes with PAA/PEI/PAA-NTA. In the future, we will use the PNTA-modified membranes for Ni²⁺ binding and His-tagged protein purification.

Chapter 4 presents my efforts to develop a lectin-containing membrane to capture glycoproteins. After adsorption of a macroinitiator, a 5-min polymerization of MES gives a membrane that binds at least 100 mg of lysozyme/cm³ of membrane. However, the binding of a glycoprotein was not extensive when Con A was immobilized in membranes using covalent or metal-affinity methods with either polymer brushes or LbL films. The low binding capacity may stem from damage to Con A, which is a pH-sensitive protein, or more likely steric hindrance to the binding sites of Con A.

Thin (~100 μm) microporous membranes also provide a unique platform for creating enzyme reactors. Chapter 5 describes development of membrane reactor containing covalently anchored trypsin. The porous membrane modified with one layer of PAA captures 35 mg of trypsin/cm³ of membrane and affords digestion rates 75 times those

for a typical in-solution digestion. In addition, the covalent immobilization stabilizes the enzyme in 6 M urea or at pH 5 to enable denaturation of proteins that typically resist digestion in their native state. These trypsin-containing membranes have a room-temperature shelf life of over 6 months. A miniature membrane holder and syringe pump afford control over digestion times and, hence, peptide lengths.

6.2. Future work

6.2.1. His-tagged protein purification using PNTA-containing membranes

Chapter 3 describes the Cu^{2+} -binding and leaching studies for membranes modified with PNTA-containing films. The data showed much less Cu^{2+} leaching compared to a PAA/PEI/PAA-NTA-modified membrane. In the next step, we will investigate Ni^{2+} binding and leaching on the PNTA-containing membranes. Further we will examine His-tagged protein binding specificity and recovery during isolation of His-tagged proteins from cell lysates or protein mixtures.

6.2.2. Immunoaffinity purification with antibody-containing membranes

Chapter 4 describes my work to develop a lectin-containing membrane to isolate specific glycoproteins. Unfortunately, these membranes have a low glycoprotein binding capacity. However, we still can expand the membrane-based technique for immunoaffinity purification using more specific antibody-antigen interactions.

Figure 6.1 shows typical bead-based immunopurification strategies. Incubation of either a free or immobilized antibody with the target lysate leads to specific binding of antigen, and subsequent capture of antibody-antigen complexes on beads followed by

elution and analysis allows detection of the antigen and its associated proteins. For example, Nicol and coworkers quantified several lung-cancer biomarker candidates with low ng/mL concentrations using enrichment with hydrazide resins modified with multiple antibodies (1). However, these bead-based pathways require long incubation times that lead to sacrifice for throughput (2). In addition, they suffer from nonspecific binding due to the low binding capacity ($< 1 \text{ mg/mL}$) (3).

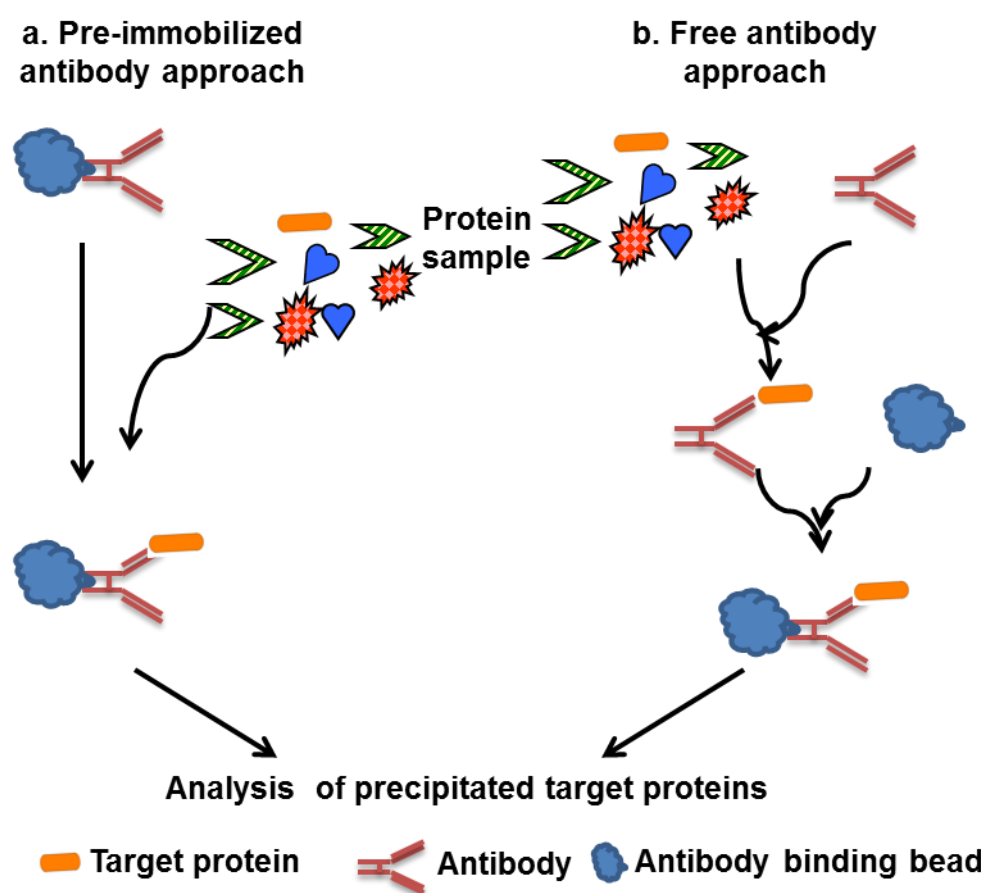


Figure 6.1. Work flow of immunoaffinity purification of target proteins using either (a) pre-immobilized antibody or (b) free antibodies that are eventually captured by antibody-binding resins. Both the pathways require hours of incubation time.

Similar to the binding of His-tagged protein, membranes should provide an attractive alternative to beads for rapid immunoaffinity capture. In preliminary studies, I

immobilized human IgG to a membrane through electrostatic interactions, but the antibody leached from the membrane. Moreover, direct covalent immobilization to a PAA-containing membrane immobilized < 5 mg of antibody/cm³ of membrane. Most recently, I employed a two-step immobilization strategy, electrostatic adsorption followed by covalent crosslinking, to immobilize IgG to the PAA/PEI/PAA modified membrane (**Figure 6.2**) (4). With this strategy, I immobilized ~ 40 mg of antibody/cm³ of membrane and also prevented antibody leaching. The IgG-containing membrane captures 7 mg of anti-IgG/cm³ of membrane, which is an order of magnitude higher than typical capacities of commercial resins (3).

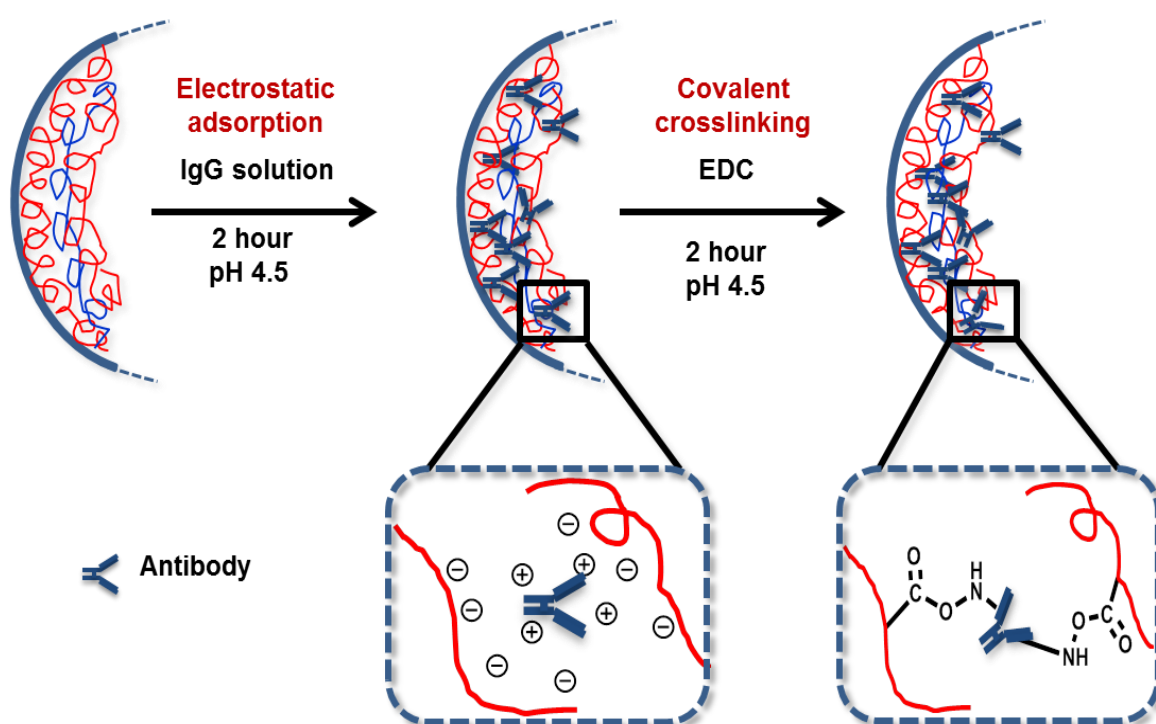


Figure 6.2. Two-step immobilization of IgG to a PAA/PEI/PAA-modified membrane.

At pH 4.5, IgG is positively charged and binds to -COO^- groups in the PAA/PEI/PAA film. IgG and EDC were dissolved in separate 20 mM phosphate solution at pH 4.5 at concentrations of 1 mg/mL and 5 mM, respectively. The solutions were circulated through the membrane (area of 3.1 cm^2) at 1 mL/min.

This high level of IgG immobilization may introduce steric hindrance, so future studies should optimize the amount of IgG bound to the membrane to achieve maximum anti-IgG binding capacity. Immobilization of multiple antibodies is possible for multiple target protein capture.

6.2.3. Probing protein structures using limited protein digestion with the trypsin membrane reactor

Chapter 5 presents the development of a trypsin membrane reactor. With a syringe pump and a miniature membrane holder, we can control the digestion time and, hence, peptide lengths. Through a statistical analysis of proteolytic events and protein 3D structures, Kazanov and coworkers ranked the importance of the factors affecting proteolysis as exposure > flexibility > local interactions (5). As a result, if a protein passes through a membrane at a high flow rate, exposed, flexible sites have the highest probability of cleavage (**Figure 6.3**). The exposed sites depend on the protein conformation, so in principle we can use limited digestion coupled with MS to probe protein conformational changes that may occur when protein binds a ligand, such as a drug (6-8).

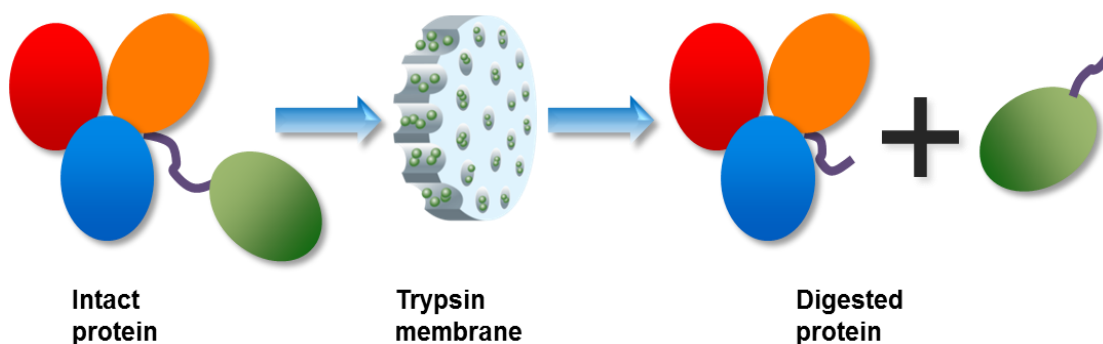


Figure 6.3. Limited digestion at the most exposed site of a protein during rapid passage through a protease-containing membrane. (The protein is not drawn to scale, as it is much smaller than the membrane pores.)

6.3. Potential impact

The membrane-modification techniques described in this dissertation provide new

devices for protein purification and digestion. The PAA/PEI/PAA-NTA-Ni²⁺ modified membrane technology is in the final stage of licensing, and new membrane devices will require only 5 min for the entire His-tagged protein purification process. Adsorption of PNTA in membranes should simplify the membrane modification to further decrease the cost of membrane-based purification of His-tagged proteins. Rapid protein associations with newly developed antibody-containing membranes should enhance immunopurification and may improve applications such as detection of biomarkers.

The protein-digestion membranes discussed in chapter 5 contain high concentrations of trypsin within membrane pores and can digest proteins in ms. Using the trypsin membrane reactor, we can control protein digestion to improve sequence coverage and PTM analysis by MS. Furthermore, we may use the limited digestion to probe changes in protein structure due to events such as drug binding. Such a technique may help elucidate drug binding sites. Overall, new methods for protein capture and immobilization in membranes should fill an important niche for rapid purification and sample preparation in biotechnology.

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