

ABSTRACT

ADAMS, VERONICA LYNN. Anion Exchange Membrane Production for Protein Molecular Weight Binding Studies. (Under the direction of Dr. Ruben Carbonell.)

Nonwoven fabric membranes are an alternative to the commonly used chromatography columns in the biomanufacturing industry. Nonwovens are an ideal material for protein separations due to their ability for high throughput with low pressure drop and to be functionalized with a variety of ligands for product capture. They also have the potential for being used as single-use technology due to their low cost. In this study, poly (glycidyl methacrylate) was grafted to the surface of poly(butylene terephthalate) nonwovens using ultraviolet (UV) graft polymerization. The extent of polymerization was measured by percent weight gain (w/w%). Following grafting, diethyl amine (DEA) was reacted to the poly(GMA) to produce a positively charged membrane for anion exchange. The membranes were challenged with three different proteins, soybean trypsin inhibitor, lipase and bovine serum albumin (BSA), to determine if protein molecular weight impacts the rate of binding in anion exchange nonwoven systems. The results show that while protein molecular weight is a key factor in static binding capacity, it is more likely that overall protein charge has a stronger effect on how well these proteins bind to the nonwoven anion exchange membranes.

Anion Exchange Membrane Production for Protein Molecular Weight Binding Studies

by
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BIOGRAPHY

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TABLE OF CONTENTS

LIST OF TABLES	iv
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	vii
INTRODUCTION AND RELEVANCE TO BIOMANUFACTURING	1
PROJECT GOALS	7
MATERIALS AND METHODS	8
Membrane Activation.....	9
Membrane Derivatization.....	9
Challenging Tertiary Ligands	10
Static Binding Capacity Quantification	11
RESULTS AND DISCUSSION	12
GMA Polymerization	12
Binding Kinetics.....	14
Static Binding Capacity versus Protein Molecular Weight.....	17
Static Binding Capacity versus Overall Protein Charge	21
CONCLUSIONS	24
PROPOSED FUTURE WORK	25
REFERENCES	26

LIST OF TABLES

Table 1: Properties of three proteins used for binding capacity study	8
Table 2: Time points for sampling during static binding study.....	11

LIST OF FIGURES

Figure 1: Poly(GMA) attached to nonwoven surface.....	4
Figure 2: Membrane surface with tertiary amine ligand (DEA) attached to poly(GMA).....	5
Figure 3: Percent weight gain for GMA graft polymerization.....	13
Figure 4: Static binding capacity for high weight gain membranes versus time.....	14
Figure 5: Static binding capacity for low weight gain membranes versus time.....	16
Figure 6: Static binding capacity versus protein molecular weight for low and high weight gain membranes.....	18
Figure 7: Static binding capacity (in mole protein/g membrane) versus protein molecular weight for low and high weight gain membranes.....	20
Figure 8: Static binding capacity versus overall protein charge for low and high weight gain membranes.....	21
Figure 9: Static binding capacity versus protein molecular weight normalized by protein charge for low and high weight gain membranes.....	23

LIST OF ABBREVIATIONS

BP	Benzophenone
BSA	Bovine serum albumin
DEA	Diethyl amine
DEG	Diethylene glycol
FTIR	Fourier transform infrared spectroscopy
GMA	Glycidyl methacrylate
PBT	Polybutylene terephthalate
pI	Isoelectric point
SEM	Scanning electron microscopy
STI	Soybean trypsin inhibitor
THF	Tetrahydrofuran
UV	Ultraviolet

INTRODUCTION AND RELEVANCE TO BIOMANUFACTURING

Industrial protein biotherapeutics are currently purified using ion exchange or affinity chromatography with packed bed resins. These resins use porous particles to separate and purify proteins, but have diffusional limitations, high cost and high pressure drop. These limitations reduce protein binding capacity and throughput, reducing efficiency of these unit operations in the purification process. For these reasons, the industry is considering other methods or materials for protein purification to replace packed bed chromatography.^{1,2}

There are several other options that could replace packed bed chromatography for protein purification. These include bulk, field-based and adsorptive separations. Bulk separations include phase extractions and partitioning, precipitation and crystallization. Precipitation and crystallization are the most commonly used methods of separation besides packed-bed chromatography. Field based separations are a variation on ultrafiltration and employ high performance tangential flow filtration methods to purify proteins. Adsorptive separations include monoliths and membrane chromatography. Membrane chromatography has been shown to be a favorable choice in bioseparations due to their ability to purify larger molecules with higher binding capacities than standard packed bed columns. This method has been adopted in some industrial applications, but still needs significant scale-up and resolution improvements before it becomes as common as packed-bed chromatography.¹

This study examines nonwoven fabric membranes as a method for protein separations in place of column chromatography. Some examples of nonwovens include nylon,

polypropylene and poly(butylene terephthalate). These nonwoven membranes are inexpensive and can be prepared with controlled porosity and fiber sizes.³ Such controllable features can be used to limit the pressure drop usually found in column chromatography. This can potentially increase throughput of protein separation processes. Also, due to the low cost of nonwoven materials, it is possible that these membranes could be used for single-use, disposable unit operations in the future. Traditionally, stainless steel or glass chromatography units are used in bioseparations for their chemical resistance and durability, but it requires additional time for often costly sanitization steps. Depth and viral filters are unit operations that are considered single-use operations and have been in use since the 1970s.⁴ The availability of single-use technology using nonwovens for traditionally non-single-use unit operations in the biomanufacturing industry, including affinity and ion exchange chromatography, may lead to higher throughput, lower cost and reduced risk of contamination. Additionally, the flexibility of single-use membrane components is ideal for pilot plants or clinical trials, offering flexible methods of changeover in confined areas or clean rooms.⁵

There are, however, some disadvantages for the use of nonwoven membranes in production scale protein purification. These include the necessity of functionalizing the membrane to effectively perform a separation. The surfaces of nonwoven membranes are generally hydrophobic, which may cause nonspecific binding and lower purity in protein separations. This requires functionalization of the membrane before it is able to bind specific proteins and limit nonspecific binding of impurities.⁶

As mentioned previously, there are several options for nonwoven fabric membranes, but this study specifically deals with poly(butylene terephthalate), or PBT. PBT has several advantages to studies performed in the past with polypropylene nonwoven fabric. The main advantage is the elimination of the ultraviolet (UV) pretreatment step required before the UV grafting step, which will be detailed in the following paragraphs. This causes PBT to have a shorter processing time in addition to having better heat stability and tensile strength than polypropylene.⁶

PBT membranes alone cannot perform effective separations due to nonspecific binding on its hydrophobic surface. This requires the membrane to be graft polymerized with UV light to functionalize the membrane. In the case of this study, the membrane is functionalized with a positively charge ligand to produce an anion exchange membrane. The first step to functionalization is UV grafting at 365 nm with poly(glycidyl methacrylate), or poly(GMA) with benzophenone (BP) as a photoinitiator. GMA is used due to its hydrophilic nature, to counteract the hydrophobicity of the PBT membrane. Also, GMA is commonly used in traditional bead resin production, providing the same microenvironment for protein separations in the nonwoven membrane as in column separations. Poly(GMA) has epoxy groups that create a brush structure for the membrane, increasing surface area for protein binding.⁷ This is shown in Figure 1 below.

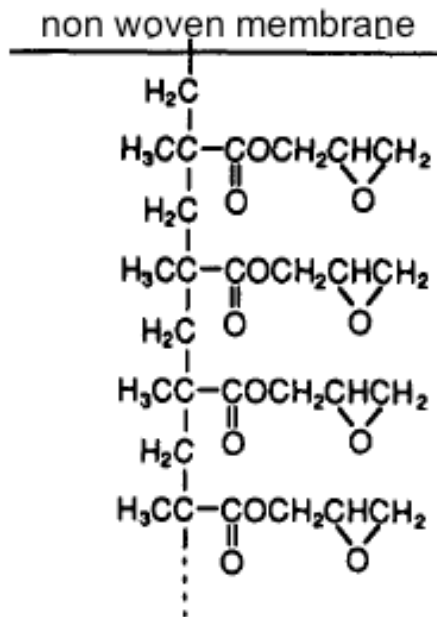


Figure 1: Poly(GMA) attached to nonwoven surface⁸

There are several variables that affect grafting, including GMA concentration, BP concentration and UV exposure time. The extent of polymerization can be characterized by weight gain analysis, SEM and FTIR. The epoxy groups can then be used to attach other ligands to the membrane, creating a surface for the desired separation. These include attaching peptides, hydroxyls, carboxyls and amines.⁹

For this study, once the PBT membranes are grafted with poly(GMA), diethyl amine (DEA) can be reacted with the epoxy groups in a ring opening reaction to form positively charged tertiary amine ligands, giving the strong anion exchange properties of the membrane. The molecular structure of a poly(GMA) modified with DEA is shown in Figure 2 below.

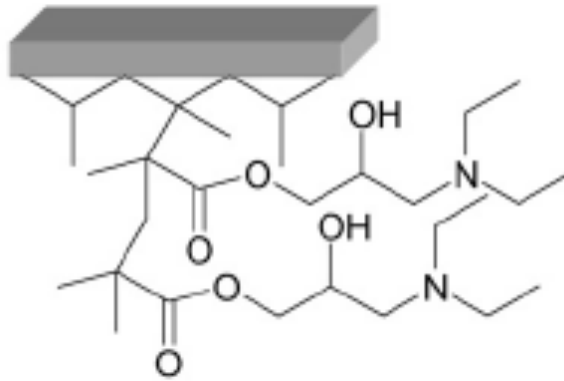


Figure 2: Membrane surface with tertiary amine ligand (DEA) attached to poly(GMA)⁶

There are two transport mechanisms to protein adsorption in nonwoven membrane separations. The first is simply binding based on charge, which is the fastest and initial transport mechanism in binding. The negatively charged protein is captured by the positively charged tertiary amine functional groups. The second transport mechanism is diffusional binding. This is significantly slower than the initial binding based on charge because its driving force is based on protein size and shape. These factors affect how the protein diffuses into the polymer space of the membrane. As protein molecular weight increases, the protein's diffusion coefficient decreases.¹⁰ This means with larger proteins the diffusion into the polymer space of the nonwoven membrane will be slower than proteins with smaller molecular weights.

Previously, nonwoven membranes have been used to capture bovine serum albumin (BSA) via anion exchange. This involved polypropylene surface modification with poly(GMA) with

an addition of a DEA ligands, diethylene glycol (DEG) ligands and a combination of DEG and DEA ligands.⁶ The drawback to using polypropylene as the nonwoven is that it requires a UV pretreatment, which is not required with PBT, which can be modified with the same poly(GMA) and tertiary amine ligands.¹¹ This study with polypropylene showed high protein binding capacities with BSA using poly(GMA) grafting and these different anion exchange ligands.⁶ Other studies have been conducted to determine the effects of UV exposure and BP concentration on surface grafting with poly(GMA) on polypropylene. It was concluded that there are limits on the effective concentrations of poly(GMA) and BP initiator to facilitate grafting onto the membranes.⁷

Instead of varying tertiary amine ligands, UV exposure and initiator concentration, this study focuses on the binding differences between proteins of varying molecular weight. UV exposure, poly(GMA) concentration and BP concentration are all held constant while three separate proteins are tested for their static binding capacities. This is to determine the initial and diffusional binding differences between proteins of varying molecular weights. The proteins tested were soybean trypsin inhibitor, lipase and BSA. These three proteins have similar isoelectric points, near 4.5. The main differences between these proteins are their molecular weight and their overall protein charge. Based on diffusional properties, it is expected that proteins with a larger molecular weight would have lower static binding capacity due to the increased difficulty of diffusion binding.

PROJECT GOALS

1. To learn how to prepare anion exchange membranes via graft polymerization and tertiary ligand attachment.
2. To run static binding capacity experiments to challenge the prepared membranes.
3. To quantify, analyze and manage the data from the static binding capacity experiments.
4. To supplement previous studies on nonwoven membrane systems.
5. To determine if protein molecular weight impacts the static binding capacity in anion exchange nonwoven systems.

MATERIALS AND METHODS

The PBT nonwoven was purchased by MacoPharma (Tourcoing, France), and has a density of 52 g/m². It has a mean pore size of 8 µm and porosity of 85%. Glycidyl methacrylate (GMA) monomer was purchased from Sigma-Aldrich (St. Louis, MO). The GMA inhibitor was removed before grafting by a pre-packed inhibitor remover (Sigma, St. Louis, MO). Benzophenone was also purchased from Sigma-Aldrich (St. Louis, MO). Tris hydrochloride, 1-butanol (A.C.S. grade), tetrahydrofuran (THF) and diethyl amine (DEA) were purchased from Fisher Scientific (Suwanee, GA). Methanol and sodium chloride were purchased from VWR International (Radnor, PA). All water used was filtered using a 0.22 µm MilliQ filter from Millipore (Billerica, MA).

Trypsin inhibitor from *Glycine max* (soybean), lipase from *Thermomysces lanuginosus* and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO).

Molecular weight, pI and overall protein charge for these proteins are shown in Table 1 below.

Table 1: Properties of three proteins used for binding capacity study

Protein	MW (kDa)	pI	Overall charge[‡]
STI	22.5	4.5	-1
Lipase	45	4.4	-6.4
BSA	66.5	4.7	-12
[‡] calculated using online protein calculator ¹² using protein amino acid sequences ^{13,14,15}			

Membrane Activation

Before activation, PBT membranes were cut into 9 cm x 22.5 cm pieces and washed in methanol for 5 minutes using a Bransonic 3510 sonicator (Branson Ultrasonics, Danbury, CT) and dried for 4 hours in a chemical hood. The monomer activation solutions used were 20% and 40% (v/v) GMA in 1-butanol with benzophenone initiator in an initiator to monomer ratio I/M = 1:20. The PBT membranes were soaked in approximately 7 mL of GMA solution before being sandwiched between two glass slides from Fischer Scientific (Suwanee, GA). Two Spectroline Model SB-100PC UV lamps (Spectronics Corp, Westbury, NY) were allowed to warm up for 10 minutes prior to use. The nonwoven membranes were then graft polymerized by UV exposure at 365 nm for t=15 minutes. After UV exposure, membranes were washed in THF for 25 minutes and then in methanol for 25 minutes to remove any ungrafted GMA monomer. The membranes were allowed to dry overnight in a chemical hood before tertiary amine addition. The extent of grafting was measured by percent weight gain (w/w%) of the membranes using Equation 1.

$$\frac{\text{Weight after grafting (g)} - \text{Weight before grafting (g)}}{\text{Weight before grafting (g)}} \times 100\% = \% \text{ Weight gain (Equation 1)}$$

Membrane Derivatization

Each membrane was derivatized with tertiary amine by the addition of 50% DEA in water. The PBT-GMA membranes were shaken in the solution for 24 hours at 90 rpm at

temperature of 30°C using a Sartorius Certomat® RM benchtop shaker (Bohemia, NY). The tertiary amine ligand was attached by a ring-opening reaction with the epoxy and DEA. The membranes were then washed 5 times in water for 5 minutes to remove any additional DEA solution. The membranes were then allowed to dry in a chemical hood overnight.

Challenging Tertiary Ligands

A total of twelve membranes were prepared. Four were selected for the static binding capacity study to challenge the ligands previously attached. Two membranes of similar weight gain were selected from each of the 20% and 40% GMA membranes. This allowed for two membrane replicates per the two GMA monomer solutions (20% and 40%) for each of the three proteins tested. Three 0.03 g samples from each of the four membranes were measured into 15 mL Falcon tubes. The samples were equilibrated with 5 mL of 20 mM Tris HCl, pH 7.2 and rotated for 30 minutes on a Glas-Col rotator (Terre Haute, IN). After equilibration, the each of the three samples was challenged with one of three different proteins: soybean trypsin inhibitor (STI), lipase and BSA. 6 mL of protein was added to each tube at a concentration of 3 mg/mL. All proteins were diluted in 20 mM Tris HCl, pH 7.2. This pH is higher than the isoelectric point for all three proteins, ensuring they are all negatively charged for the separation. The tubes with membrane and protein were placed on the rotator for a 24-hour static binding study. 50 µL samples were taken periodically over the 24-hour period, as shown in Table 2 below.

Table 2: Time points for sampling during static binding study

Time Point	Minutes	Hours
1	0	0.00
2	11	0.18
3	21	0.35
4	31	0.52
5	50	0.83
6	70	1.17
7	108	1.80
8	155	2.58
9	230	3.83
10	315	5.25
11	435	7.25
12	1290	21.50
13	1485	24.75

Static Binding Capacity Quantification

Static binding capacity for each protein on the anion exchange membranes can be determined by both indirect and direct methods. Binding capacity was measured indirectly over the 24-hour study by differential analysis of the remaining protein solution in the Falcon tubes.

Binding capacity can also be measured directly by quantifying the protein bound to the membrane by elution at the end of the study. After the 24-hour period, the falcon tubes were centrifuged in an IEC Centra CL2 from Thermo Scientific (Rockford, IL). The supernatant in the centrifuge tubes was decanted and washed with 5 mL 20 mM Tris HCl and this process repeated three times. The protein was then rotated for 40 minutes in 5 mL 20 mM Tris HCl + 1 M NaCl, pH 7.2 for elution.

Protein concentrations for indirect sampling methods were quantified by bicinchoninic acid (BCA) assay. The Pierce BCA assay kits were purchased from Thermo Scientific (Rockford, IL). The Costar 3635 plates used for the assay were purchased from Corning Incorporated (Corning, NY). The vendor provided the methods used for the assay and the plates were incubated at room temperature for 2 hours. After incubation, plates were read on a μ Quant plate reader from Bio-tek Instruments Incorporated (Winooski, VA). Using the concentrations given in the output of the plate reader, the static binding capacities at each time point for indirect measurements were calculated using Equation 2.

$$\frac{\left[\text{Beginning protein conc.} \left(\frac{\text{mg}}{\text{mL}} \right) - \text{End protein conc.} \left(\frac{\text{mg}}{\text{mL}} \right) \right] \times \text{mL protein volume}}{\text{g membrane}} \quad (\text{Equation 2})$$

$$= \text{Static binding capacity} \left(\frac{\text{mg protein}}{\text{g membrane}} \right)$$

RESULTS AND DISCUSSION

GMA Polymerization

Extent of GMA polymerization on the PBT membranes was quantified by percent weight gain. Membranes treated with 20% GMA solution (low weight gain membranes) had a percent weight gain ranging from 2.80 to 4.90%, with a mean of 3.61 ± 0.761 . Membranes treated with 40% GMA solution (high weight gain membranes) had weight gains ranging from 6.08 to 7.74%, with a mean of 6.94 ± 0.679 . These values are shown in Figure 3 below.

With higher GMA concentration, the membranes had higher weight gain due to the additional GMA available for polymerization. The weight gain results were consistent with what was expected.

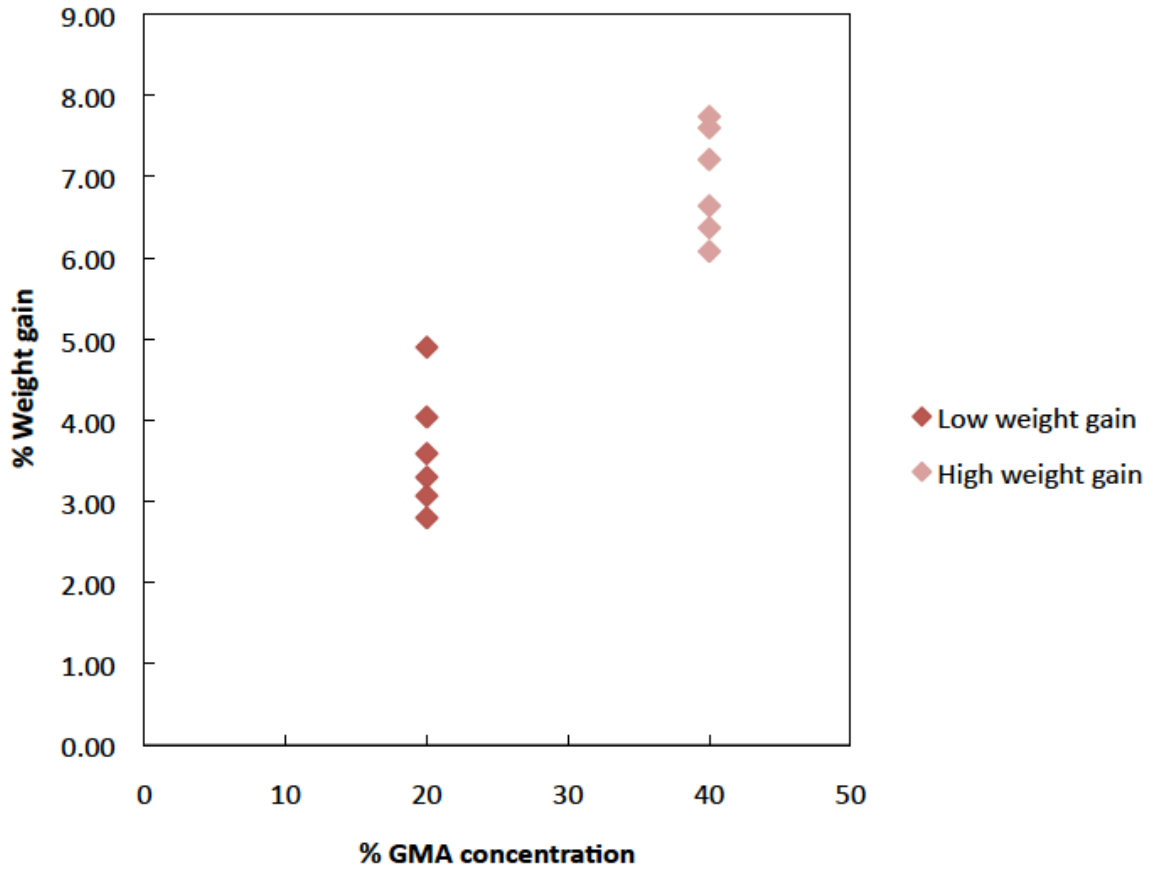


Figure 3: Percent weight gain for GMA graft polymerization

Binding Kinetics

Calculated static binding capacity values were plotted against time for the 24-hour binding study. Average values for the two high weight gain membrane replicates (7.60, 7.74%) are shown below in Figure 4 with standard error bars (n=2), comparing static binding capacities for STI, lipase and BSA.

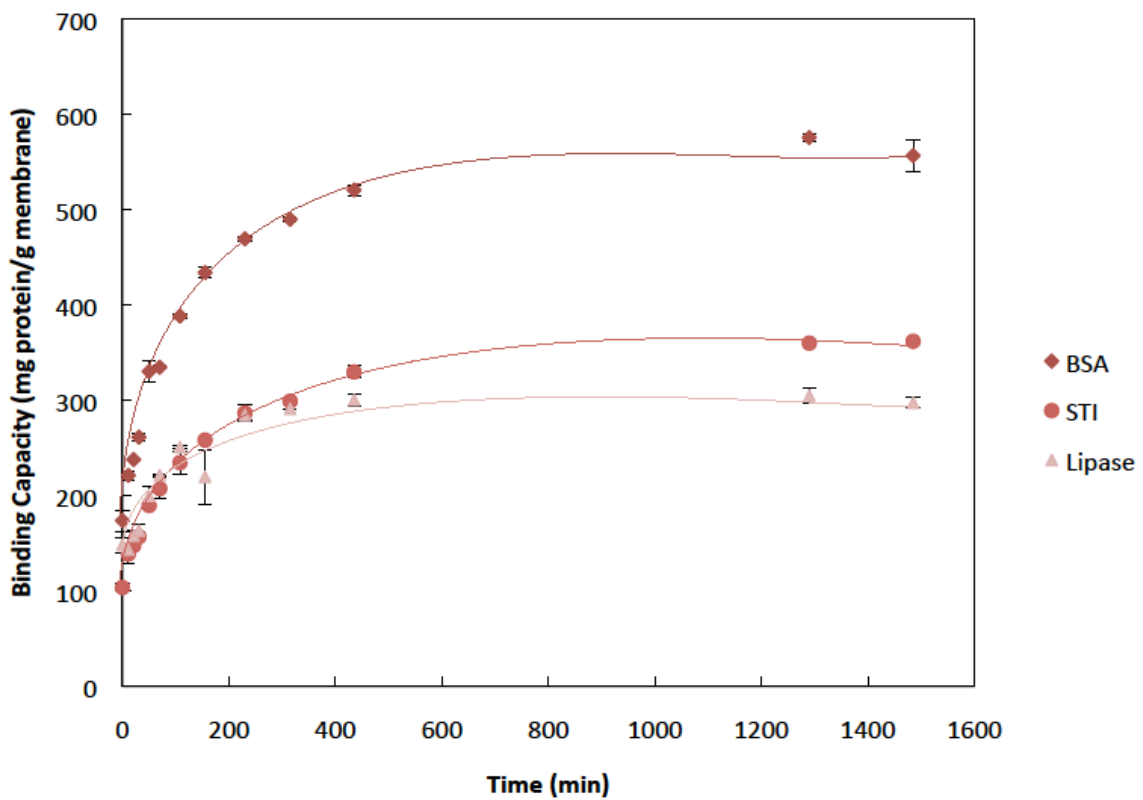


Figure 4: Static binding capacity for high weight gain membranes versus time

Initial charge binding for the high weight gain membrane continues until time point 435, or 7.25 hours after beginning the study. After this point, the transport mechanism for binding is diffusion and increases slowly to the equilibrium binding value. The binding capacities after 24 hours were 301 mg/g, 361 mg/g and 566 mg/g for lipase, STI and BSA, respectively. STI and lipase had almost the same static binding capacity for the high weight gain membranes, but BSA was significantly higher. The initial charge binding was also faster for BSA, possibly due to its higher overall protein charge. All three proteins have very similar isoelectric points, so the faster initial binding is likely due to the higher negative charge of the BSA (-12e) causing it to bind more quickly to the anion exchange membrane. Lipase has only half of the negative charge (-6.4e) as BSA and STI is significantly lower (-1e).

Calculated static binding capacity values were also plotted against time for the 24-hour binding study for the low weight gain membranes. Average values for the two low weight gain membrane replicates (3.07, 3.30%) are shown below in Figure 5 with standard error bars (n=2), comparing static binding capacities for STI, lipase and BSA.

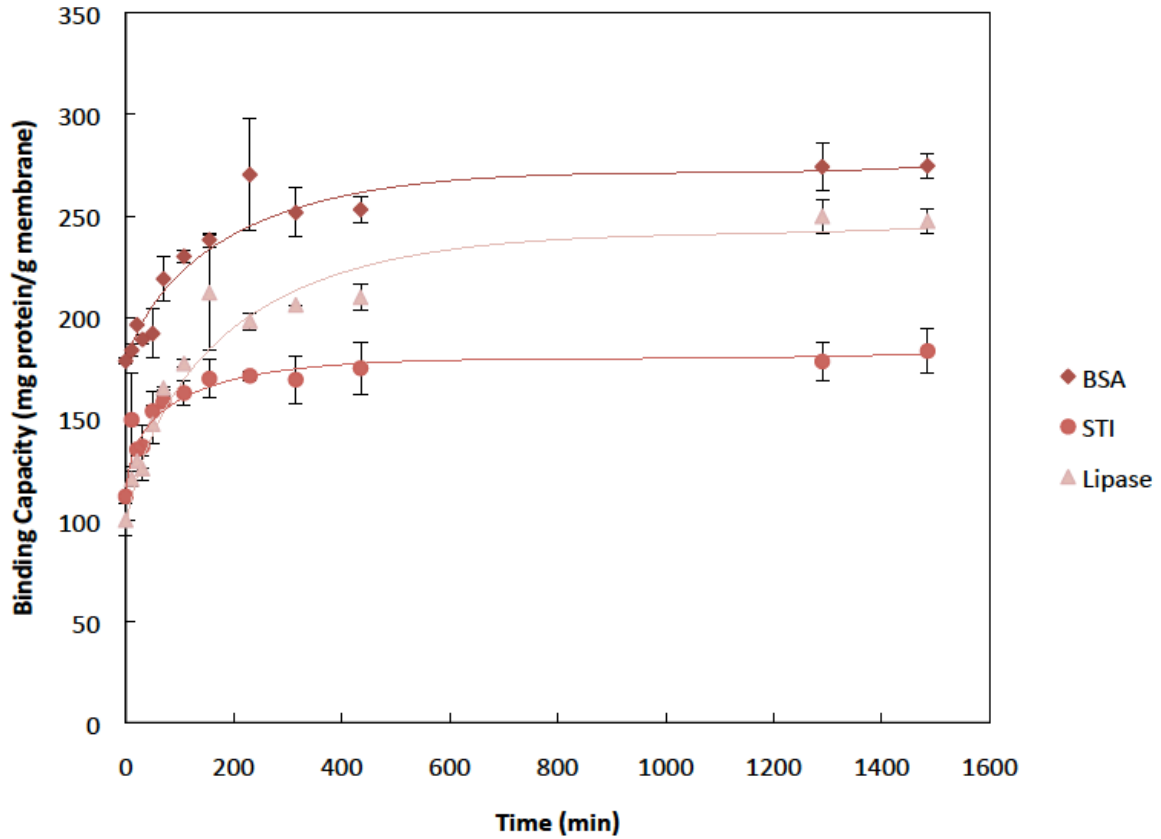


Figure 5: Static binding capacity for low weight gain membranes versus time

Initial charge binding for the low weight gain membrane continues until time point 315, or 5.25 hours after beginning the study. After this point, the binding transport mechanism is diffusion and increases slowly to the equilibrium binding value. The binding capacities after 24 hours were 181 mg/g, 249 mg/g and 274 mg/g for STI, lipase and BSA, respectively. For the low weight gain membranes, static binding capacity increased with protein size. This result seems to be counterintuitive to the expected result of higher binding capacity for

smaller proteins, but it is possible that the binding capacities are significantly affected by the overall charge of the protein rather than the molecular weight. In this case, the protein with the highest overall negative charge should have the highest binding capacity, which is true for this study. All three proteins have very similar initial binding rates, unlike the high weight gain membranes. The initial rate of binding in the low weight gain membranes is also lower than the high weight gain membranes. This could be due to the lower weight gain membrane having less tertiary amine ligands available to facilitate strong initial binding.

Static Binding Capacity versus Protein Molecular Weight

Equilibrium static binding capacities for each protein on both high and low weight gain membranes were plotted against protein molecular weight. This was to compare the binding capacity differences for the high and low weight gain membranes based on protein size. This is shown in Figure 6 below.

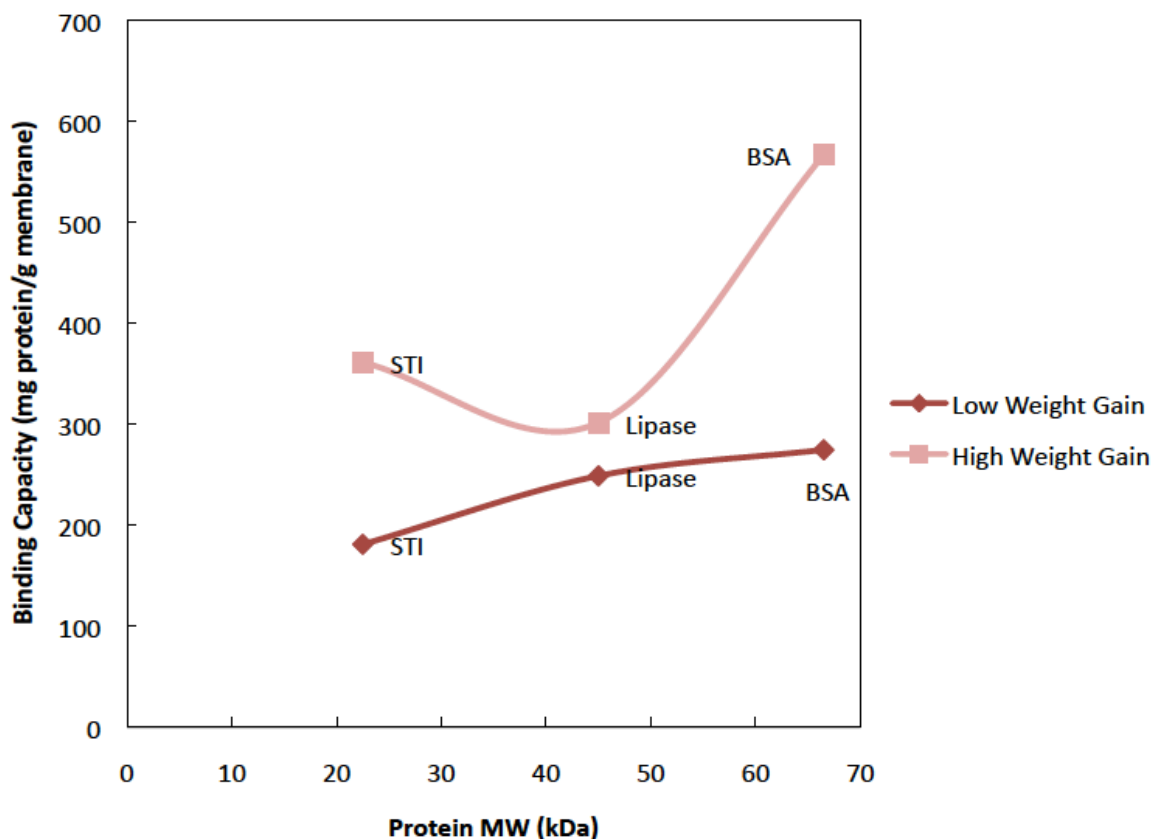


Figure 6: Static binding capacity versus protein molecular weight for low and high weight gain membranes

The high weight gain membranes have the largest differences in binding capacities for the different molecular weight proteins, while the low weight gain binding capacities increase almost linearly with protein molecular weight. It is likely these differences have to do with the positive tertiary amine ligands available on the membrane and also the overall charge differences for each protein. It is unexpected that lipase has a lower binding capacity than

STI since it is a larger protein and the other trends suggest that binding capacity increases with protein size. This was seen in the binding kinetics for lipase and STI as well, and can be assumed that because BSA had such a higher binding capacity than these two that they are not significantly different values.

All static binding capacities up to this point have been calculated by mg protein bound to g nonwoven membrane, as this is the common way to report binding capacity in industry.

Another way to interpret binding capacity is to calculate moles of protein bound per g membrane, especially to compare proteins with different molecular weights. By reporting binding capacity in terms of mg protein per g membrane, the binding capacity is directly linked to the protein's molecular weight, which causes larger proteins, like BSA, to have a higher binding capacity than a smaller protein, like STI, even if the same amount of protein molecules bind to the membrane. Using moles of membrane bound to the membrane, it is possible to remove the molecular weight variable in protein binding capacity. This interpretation is shown in Figure 7 below.

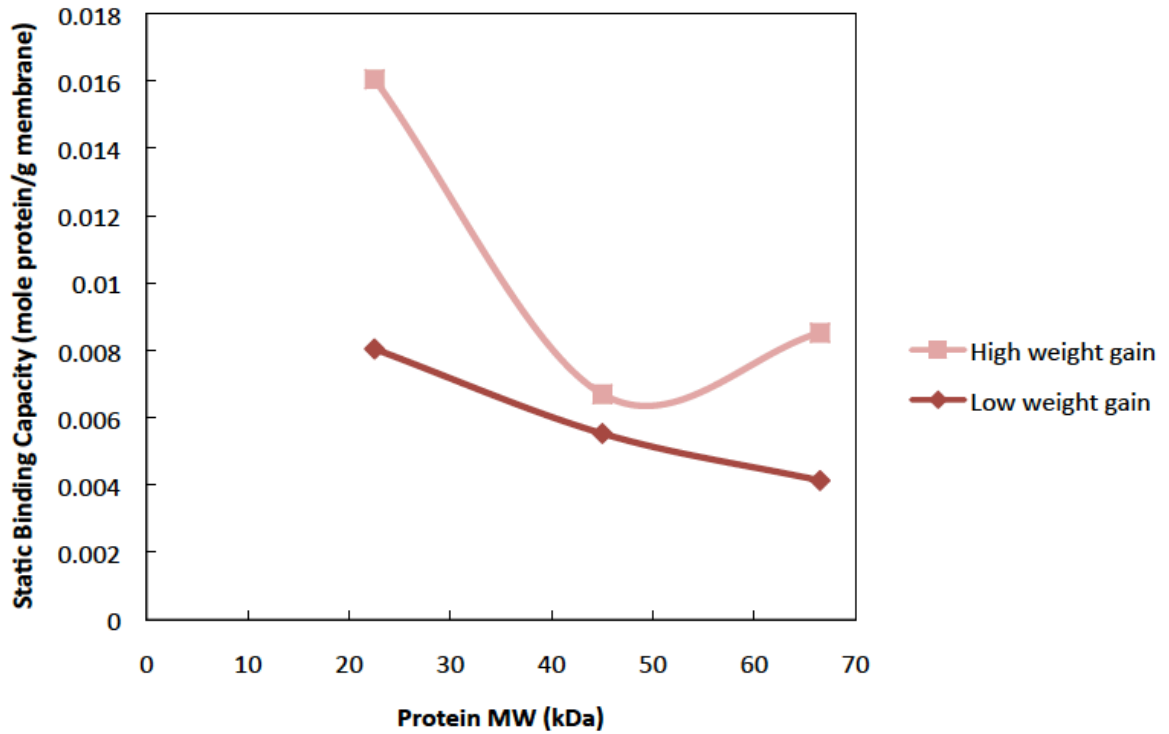


Figure 7: Static binding capacity (in mole protein/g membrane) versus protein molecular weight for low and high weight gain membranes

By plotting binding capacity using moles of protein bound per membrane, the graph is mirrored to that of the previous plot in Figure 6. Static binding capacity based on moles of protein decreases with protein molecular weight, which was the original expectation of this study. Industry, however, reports binding capacities in mg or g of protein bound, so the remainder of plots in this study are reported in that way.

Static Binding Capacity versus Overall Protein Charge

Equilibrium static binding capacities for each protein on both high and low weight gain membranes were also plotted against overall protein charge. This was to determine if there were any additional trends in binding capacities between the high and low weight gain membranes based on overall protein charge. This is shown in Figure 8 below.

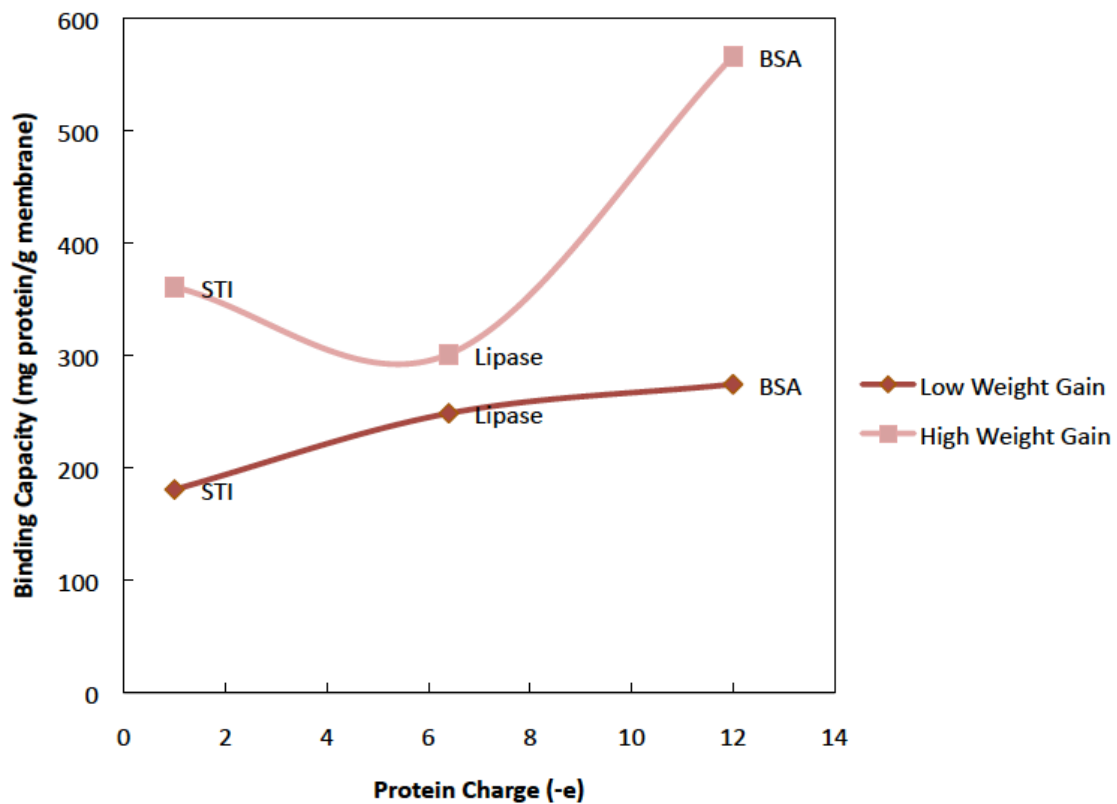


Figure 8: Static binding capacity versus overall protein charge for low and high weight gain membranes

Plotting against overall protein charge shows a very similar trend as plotting against protein molecular weight. This suggests that overall protein charge likely has a significant effect in protein binding to the membrane. For both the high and low weight gain membranes, binding capacities increase with increased negative charge except for the case in the high weight gains where STI and lipase are not very different. It makes logical sense that a protein with a higher overall negative charge, even with the same isoelectric point as other proteins, would have higher affinity to a positively charged anion exchange membrane.

In an effort to normalize the protein molecular weight based on overall protein charge, the equilibrium static binding capacities for each protein on both high and low weight gain membranes were also plotted against protein molecular weight divided by overall protein charge. Normalizing by overall protein charge is an attempt to eliminate one of the variables of the experiment. This is shown in Figure 9 below.

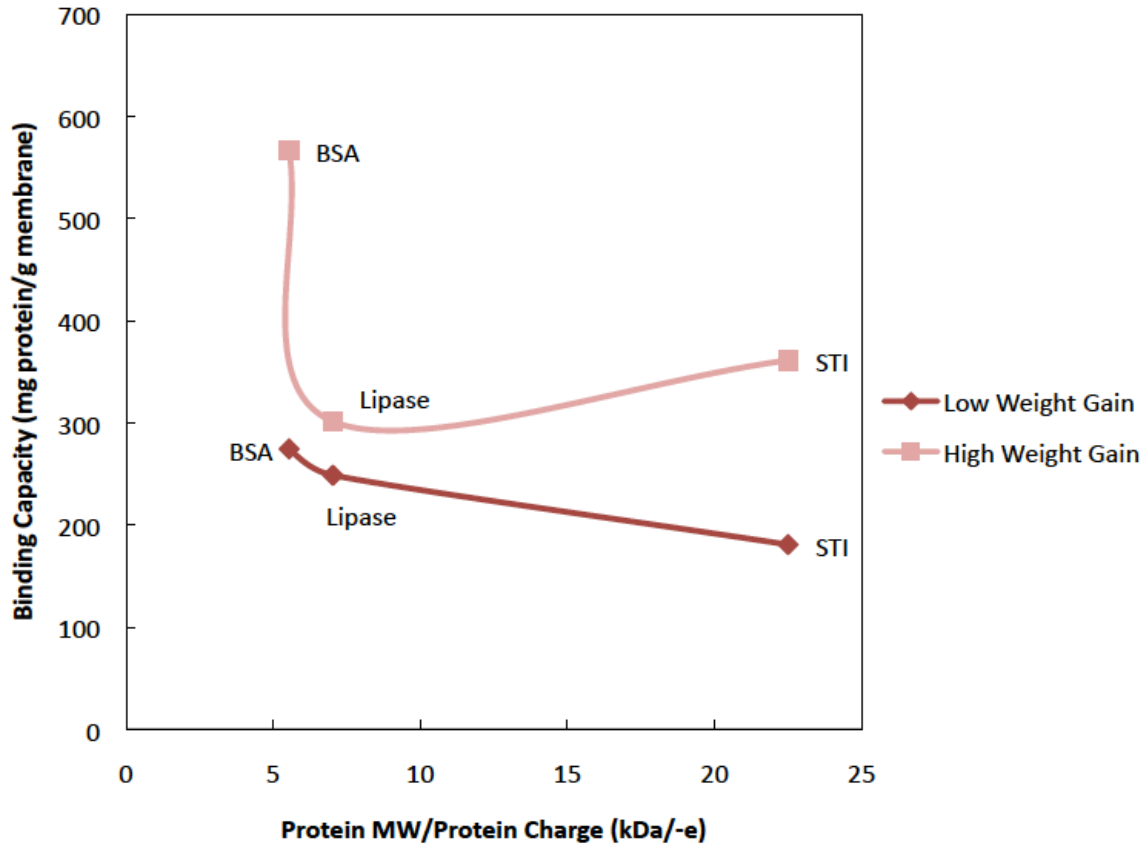


Figure 9: Static binding capacity versus protein molecular weight normalized by protein charge for low and high weight gain membranes

This plot exhibits some of the same trends as the previous plots. The most apparent difference is that binding capacity decreases with normalized protein molecular weight. This is due to the overall charges of the proteins increasing with the molecular weight of the protein, in the case of this study. It essentially shows the same information, only mirrored to that of previous plots. It continues to suggest that overall protein charge likely has a

significant effect in protein binding to the membrane when considering proteins of different molecular weights with similar isoelectric points.

CONCLUSIONS

This study analyzed the effects of GMA concentration on graft polymerization of anion exchange nonwoven membranes and the differences in static binding capacity on the different weight gain membranes using three different proteins, soybean trypsin inhibitor, lipase and bovine serum albumin. The study also examined how protein molecular weight and overall protein charge influences static binding capacity in these nonwoven systems. The study focused on both initial charge and diffusion binding.

Results indicate high static binding capacities for these three proteins that increase with protein molecular weight. While this result was not expected, the increased binding with the larger size proteins may be due to a higher overall negative protein charge. As overall negative protein charge increased, the static binding capacity increased. With a higher negative charge, it is expected that binding would be increased in a positively charged anion exchange system. When binding capacities calculated as moles of protein bound were plotted, the binding capacity decreased with protein molecular weight. While this study gives little definitive results, it does contribute some helpful information and methods that could be useful in future studies to examine the effect protein molecular weights and protein charge on binding to nonwoven systems.

PROPOSED FUTURE WORK

Studies could be conducted in the future using the same techniques, but with a wider range of protein molecular weights. This could further study the effects of protein molecular weight and overall protein charge on static binding capacities. Proteins of similar molecular weights with differing overall charge could also be examined to gain more information on the overall charge influence on binding. These future studies could also incorporate dynamic binding capacity studies in addition to the static binding conditions already used.

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