SURFACE MODIFICATION OF AN ACRYLATE-BASED SHAPE MEMORY POLYMER TO PROMOTE ADSORPTION TO BIOMARKERS OF EOSINOPHILIC ESOPHAGITIS

by

ROOPALI R. SHAH

B.A., University of Colorado Boulder, 2008

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment of the requirements for the degree of
Masters of Science
Bioengineering
2013
This thesis for the Masters of Science degree by

Roopali R. Shah

has been approved for the

Bioengineering Program

by

Robin Shandas, Chair
Glenn Furuta, Advisor
Dae Won Park, Advisor

June 19, 2013
Shah, Roopali, R (M.S., Bioengineering)

Surface Modification of an Acrylate-Based Shape Memory Polymer to Promote Adsorption to Biomarkers of Eosinophilic Esophagitis

Thesis directed by Professor Robin Shandas

ABSTRACT

The surface of an acrylate-based shape memory polymer ("SMP") was modified to capture cationic biomarkers of eosinophilic esophagitis ("EoE") to establish a better diagnostic and monitoring system of this disease. Incorporation of a negative charge into the SMP allows for specific adsorption of the cationic biomarkers with additional leverage from the shape memory effect to position the polymer near the lumen wall of the esophagus. SMP modification was performed by copolymerization with acrylate acids at increasing weight percentages to incorporate a negative, electrostatic charge onto the polymer surface, but still preserve and instill hydrophobic interactions. Because literature suggests that electrostatic, hydrophobic and hydrogen bonds are the major driving interactions for adsorption, the polymer was the target of modification to the system in the initial phases of the experimental design. Reproducible binding did not occur with in-vivo mimicking systems. Polystyrene microspheres, a commercially available positive control known to adsorb proteins, was introduced to the experimental process after controlling for other influential adsorption parameters (pH, protein, protein concentration, surface-to-volume ratios, pH, riddance of impurities or competing proteins, etc.) that also established no binding to the surface of the SMPs. The positive control facilitated a checkpoint for the development of SMP adsorption. The microspheres were used to check against all experimental parameters that were performed prior, to measure encouraging or discouraging protein adsorption factors. The polystyrene microspheres unveiled the importance of ionic strength, pure protein solutions and surfactants to adsorption. This illustrates that adsorption depends on multiple factors and that electrostatic charge and hydrophobicity on the polymer surface is just one such parameter. The system was revised and binding was performed with SMPs incorporated with 0, 0.5, 1 and 2.5 wt% of SEM. Results showed significant amounts of protein binding onto the surface of the SMP’s. Additionally, adsorption of the SEM polymers was tested against the adsorption of polystyrene and nylon for comparative analysis of mainstream biomedical materials. SEM
SMPs bind to protein preferentially over these materials suggesting our SMPs are better adsorbents, paving the way for many biomedical applications.

The form and content of this abstract are approved. I recommend its publication.

Approved: Robin Shandas
ACKNOWLEDGMENTS

I would like to thank Kiran Dyamenahalli, Dr. Steve Ackerman and my committee members for their time and helpful guidance through this process. A special thanks to my parental unit, Raj and Geeta Shah for their encouragement and endless support.
# TABLE OF CONTENTS

## Chapter

1. Introduction ...................................................................................................................................................... 1

1.1 Shape Memory Polymer: A Diagnostic Approach for Eosinophilic Esophagitis ........................................ 1

2. Background ....................................................................................................................................................... 3

2.1 Eosinophilic Esophagitis and the Esophageal String Test ................................................................................. 3

2.2 Adsorption Principles ...................................................................................................................................... 4

2.3 SMPs and Surface Modification .................................................................................................................. 6

3. Rational for the Experimental Design and Important Considerations for Adsorption Studies ..................... 8

3.1 Prelude to Rationale ........................................................................................................................................ 8

3.2 Polymer .......................................................................................................................................................... 8

3.2.1 Major Interactions Forces Involved in Adsorption ....................................................................................... 8

3.2.2 Shape Memory Polymer .......................................................................................................................... 10

3.2.3 Surface Area .............................................................................................................................................. 10

3.2.4 Impurities on the Polymeric Surface ......................................................................................................... 10

3.2.5 Post Processing ......................................................................................................................................... 11

3.3 Protein ............................................................................................................................................................ 13

3.3.1 General Considerations for the Study Protein .......................................................................................... 13

3.3.2 Poly-L-Arginine ....................................................................................................................................... 14

3.3.3 Eosinophil Derived Granule Proteins .................................................................................................... 14

3.3.4 Bovine Serum Albumin .......................................................................................................................... 15

3.4 Solution ......................................................................................................................................................... 15

3.4.1 Solution’s Importance ............................................................................................................................ 15

3.4.2 Temperature ............................................................................................................................................. 15

3.4.3 pH ............................................................................................................................................................. 16

3.4.4 Ionic Strength ........................................................................................................................................... 16
3.4.5 Purity ................................................................................................................. 16

4. Materials and Methods .......................................................................................... 17
4.1 Materials ............................................................................................................... 17
4.2 Methods ................................................................................................................ 17
4.2.1 Characterization ............................................................................................... 17
4.2.1.1 Contact Angle and Surface Free Energy ..................................................... 17
4.2.1.2 pH Testing .................................................................................................. 18
4.2.1.3 Water Equivalency Test .............................................................................. 18
4.2.1.4 Dynamic Mechanical Analysis ................................................................. 18
4.2.1.5 Fourier Transform Infrared Analysis ......................................................... 19
4.2.2 Adsorption Testing ......................................................................................... 19
4.2.2.1 SMP Synthesis ......................................................................................... 19
4.2.2.2 Binding Experiments .............................................................................. 19
4.2.2.3 Experiment 1. MA .................................................................................. 20
4.2.2.4 Experiment 2. CEA .............................................................................. 20
4.2.2.5 Experiment 4. Surface Area 132mm²/ml .................................................... 21
4.2.2.6 Experiment 5. Surface Area with SMP Particles ...................................... 21
4.2.2.7 Experiment 6. Surface Area with SMP Particles ...................................... 23
4.2.2.8 Experiment 7. Surface Area 648mm²/ml ................................................... 24
4.2.2.9 Experiment 8. Eosinophil Lysate .............................................................. 24
4.2.2.10 Experiment 9-10. Eosinophil Lysate ....................................................... 25
4.2.2.11 Experiment 11. Eosinophil Lysate ...................................................... 25
4.2.2.12 Experiment 12. CEA ........................................................................... 26
4.2.3 Positive Control- Optimization of Polystyrene Microspheres ...................... 26
4.2.3.1 General procedure ................................................................................... 26
4.2.3.2 Surface Area and Surfactant Optimization .............................................. 27
4.2.3.3 Total Exclusion of Surfactants ................................................................. 27
## Appendix

A: Detection .......................................................................................................................... 84

A.1 Overview of Detection Methods .................................................................................. 84

A.2 Micro BCA Assay ........................................................................................................ 88

A.3 Bio-Rad Protein Assay ............................................................................................... 91

A.4 Spectroscopy .............................................................................................................. 92

A.5 Flamingo Fluorescent Gel Stain .................................................................................. 93

B: Protocols .......................................................................................................................... 96

B.1 Acetate Buffer ............................................................................................................. 96

B.2 Polystyrene Microspheres .......................................................................................... 97

B.3 SEM Binding Experiment ........................................................................................... 98

B.4 Eosinophil Lysate ....................................................................................................... 100
LIST OF TABLES

Table

1 Protein Characterization ................................................................. 14
2 Contact Angle and Surface Energy Measurements: Average of Ten Trials ........................................ 31
3 Contact Angle and Surface Energy Measurements: Average of Ten Trials ........................................ 37
4 Water Equivalency Test: Average of 2 Samples ............................................................................. 39
5 Thermal Mechanical Properties of SEM SMPs: Average of Two Trials ............................................. 42
6 Protein Characterization .............................................................................. 56
7 % Protein Bound Relative to Total ECP ............................................................................. 57
8 % Protein Bound Relative to Total EDN ............................................................................. 57
9 % Protein Bound Relative to Total MBP1 ............................................................................. 57
10 % Protein Bound Relative to Total ECP ............................................................................ 59
11 % Protein Bound Relative to Total EDN ............................................................................ 59
12 % Protein Bound Relative to Total MBP1 ............................................................................ 60
13 % Protein Bound Relative to Total ECP ............................................................................ 60
14 % Protein Bound Relative to Total EDN ............................................................................ 61
15 BSA Characterization ......................................................................................... 63
16 % Protein Bound Relative to Total ECP ............................................................................ 63
17 % Protein Bound Relative to Total BSA ............................................................................ 66
18 % Protein Bound Relative to Total BSA ............................................................................ 66
19 % Protein Bound Relative to Total BSA ............................................................................ 67
20 % Protein Bound Relative to Total BSA ............................................................................ 68
21 % Protein Bound Relative to Total BSA ............................................................................ 69
22 % Protein Bound Relative to Total BSA-488 ........................................................................ 70
23 % Protein Bound Relative to Total Poly-L-Arginine ............................................................... 71
24 % Protein Bound Relative to Total BSA ............................................................................ 73
25 % Protein Bound Relative to Total BSA ............................................................................ 76
26: Antibody-Based Assays ................................................................. 85
27: Absorbance-Based Assays ............................................................ 86
28: Fluorescence-Based Assays ............................................................ 87
29: BCA Assay with Hanks Balanced Salt Solution (HBSS) ..................... 89
30: BCA Assay with RPMI Diluted with Water (W) or PBS (P) ................... 89
31: BSA Standard Curve Diluted in PBS (1X) ........................................ 90
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Important Protein Adsorption Determinants</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Schematic of Major Adsorption Interaction</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>Schematic of Surfactants Inhibiting Protein Binding</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Methanol Treatments to CEA SMPs</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>Sterilization Treatments to SEM SMPs and Polystyrene</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>Processing of SMP Particles</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Adsorption Optimization Techniques</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td>Contact Angle and Surface Energy Measurements of CEA SMPs</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>pH Testing of CEA SMPs</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>DMA Results on CEA SMPs</td>
<td>34</td>
</tr>
<tr>
<td>11</td>
<td>FTIR on CEA SMPs</td>
<td>35</td>
</tr>
<tr>
<td>12</td>
<td>Contact Angle and Surface Energy with SEM SMPs</td>
<td>36</td>
</tr>
<tr>
<td>13</td>
<td>Water Equivalency Test with SEM SMPs</td>
<td>38</td>
</tr>
<tr>
<td>14</td>
<td>DMA Results on SEM SMPs</td>
<td>41</td>
</tr>
<tr>
<td>15</td>
<td>Thermomechanical Reproducibility Analysis</td>
<td>42</td>
</tr>
<tr>
<td>16</td>
<td>FTIR on SEM SMPs</td>
<td>44</td>
</tr>
<tr>
<td>17</td>
<td>Experiment 1 MA</td>
<td>46</td>
</tr>
<tr>
<td>18</td>
<td>Experiment 2-3. CEA</td>
<td>47</td>
</tr>
<tr>
<td>19</td>
<td>Surface Areas Implemented to Maximize Loading Capacity</td>
<td>48</td>
</tr>
<tr>
<td>20</td>
<td>Experiment 4. Surface Area 132mm²/ml</td>
<td>48</td>
</tr>
<tr>
<td>21</td>
<td>SMP Particles</td>
<td>50</td>
</tr>
<tr>
<td>22</td>
<td>Nylon Particles</td>
<td>51</td>
</tr>
<tr>
<td>23</td>
<td>Experiment 5 Surface Area with SMP Particles</td>
<td>52</td>
</tr>
<tr>
<td>24</td>
<td>Experiment 6 Surface Area with SMP Particles-Complete Submersion</td>
<td>53</td>
</tr>
<tr>
<td>25</td>
<td>Experiment 7 Surface Area 648mm²/ml</td>
<td>54</td>
</tr>
<tr>
<td>Experiment Number</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>8</td>
<td>Eosinophil Lysate. ECP Adsorption</td>
<td>56</td>
</tr>
<tr>
<td>8</td>
<td>Eosinophil Lysate. EDN Adsorption</td>
<td>57</td>
</tr>
<tr>
<td>8</td>
<td>Eosinophil Lysate. MBP1 Adsorption</td>
<td>57</td>
</tr>
<tr>
<td>9</td>
<td>Eosinophil Lysate. ECP Adsorption</td>
<td>59</td>
</tr>
<tr>
<td>9</td>
<td>Eosinophil Lysate. EDN Adsorption</td>
<td>59</td>
</tr>
<tr>
<td>9</td>
<td>Eosinophil Lysate. MBP1 Adsorption</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>Eosinophil Lysate. MBP1 Adsorption</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>Eosinophil Lysate. EDN Adsorption</td>
<td>61</td>
</tr>
<tr>
<td>11</td>
<td>Eosinophil Lysate. ECP Adsorption</td>
<td>63</td>
</tr>
<tr>
<td>12</td>
<td>CEA</td>
<td>64</td>
</tr>
<tr>
<td>12</td>
<td>Surface Area and Surfactant Optimization</td>
<td>65</td>
</tr>
<tr>
<td>12</td>
<td>Total Exclusion of Surfactants</td>
<td>66</td>
</tr>
<tr>
<td>12</td>
<td>BSA Concentration Optimization</td>
<td>67</td>
</tr>
<tr>
<td>12</td>
<td>Buffer Solution Optimization</td>
<td>68</td>
</tr>
<tr>
<td>12</td>
<td>Reproducibility Testing</td>
<td>69</td>
</tr>
<tr>
<td>12</td>
<td>BSA Alexa Fluor 488</td>
<td>70</td>
</tr>
<tr>
<td>12</td>
<td>Experiments 1-7. Poly-L-Arginine</td>
<td>71</td>
</tr>
<tr>
<td>12</td>
<td>Experiments 8-11. Eosinophil Lysate</td>
<td>72</td>
</tr>
<tr>
<td>12</td>
<td>Experiments 12. BSA and Ionic Strength</td>
<td>73</td>
</tr>
<tr>
<td>12</td>
<td>SEM Adsorption of BSA</td>
<td>75</td>
</tr>
<tr>
<td>12</td>
<td>SEM Polymer after 3 Hour Incubation</td>
<td>77</td>
</tr>
<tr>
<td>12</td>
<td>Images of the SEM Polymer after 3 Hour Incubation</td>
<td>78</td>
</tr>
<tr>
<td>12</td>
<td>Micro BCA Assay Performed on Experiment 12 CEA</td>
<td>91</td>
</tr>
<tr>
<td>12</td>
<td>Bio-Rad Assay Performed on Experiment 12 CEA</td>
<td>91</td>
</tr>
<tr>
<td>12</td>
<td>Spectroscopy Performed on Experiment 12 CEA</td>
<td>92</td>
</tr>
<tr>
<td>12</td>
<td>Spectroscopy Performed on Experiment 12 CEA</td>
<td>93</td>
</tr>
<tr>
<td>12</td>
<td>Flamingo Fluorescent Gel Stain on BSA Standards, Exposure with a Fluorometer at 532nm</td>
<td>94</td>
</tr>
<tr>
<td>12</td>
<td>Flamingo Fluorescent Gel Stain on BSA Standards, Exposure with UV Transilluminescence</td>
<td>94</td>
</tr>
</tbody>
</table>
54 BSA Standard Curve from Fluorescent Gel Stain.
1. Introduction

1.1 Shape Memory Polymer: A Diagnostic Approach for Eosinophilic Esophagitis

In developed countries, 15% of all medical cases are misdiagnosed\(^1\). *The Seattle Times* reports “nearly one-third of the 2.7 trillion spent each year on healthcare in the U.S. are considered to be wasted dollars.” The current diagnostics set in place for eosinophilic esophagitis (“EoE”), a chronic inflammatory allergic disease of the esophagus, contributes to this heavy expense. In this disease, eosinophils invade the esophagus and wear away the esophageal lining. Biopsies are the gold standard for detecting EoE and depend on measuring these eosinophils\(^2\). Since the eosinophils are not evenly distributed within the esophagus, and the procedure is limited to acquiring less than a 0.7% sample size relative to the entire esophageal surface area, the diagnosis can be missed\(^3\). One study reported a correlation between the quantity of these eosinophils and highly cationic eosinophil derived granule proteins (“EDGPs”) they secrete in the luminal mucosa\(^4\). Therefore, measuring mucosal inflammation can be one route to effectively diagnose EoE patients and is under investigation through a novel esophageal string test \(^2,4\). In this approach, a swallowable nylon string is used to capture these cationic proteins in the lumen of the esophagus. The test drives down the diagnostic costs and is minimally invasive. However, small sample sizes of the EDGP’s are an underlining issue due to small sample volume collected onto the string and the lack of specificity for the EDGPs. With these limitations of current detection methods, patients of EOE are needlessly suffering from symptoms that affect their quality of life.

The initial aim of this study was to chemically tailor a polymeric surface for the specific capture of EDGPs. This contribution can aid in the development of a minimally invasive, inexpensive and reliable diagnostic for EoE. Incorporation of anionically charged groups to the surface of a shape memory polymer (“SMP”) can selectively adsorb these unique cationic proteins with additional leverage from the SMP to position itself near the lumen wall of the esophagus in close proximity to the EDGP’s. Additionally, the shape memory effect of the polymer allows for substantial increase in recoverable surface area during deployment, correlating to capturing greater sample sizes. Adsorption mechanisms have been extensively studied and reported as a complex system, where the exact occurrences between the protein and surface interfaces are unknown\(^5-7\). However, literature states the dominating factors that affect adsorption are electrostatic, hydrogen and hydrophobic interaction forces\(^5-9\). Recent studies have found trends of
adsorption through electrostatic and hydrophobic interactions. These groups found that adsorption through negative electrostatic interactions is higher relative to adsorption through hydrophobic interactions\textsuperscript{5,7}. We therefore hypothesized that the incorporation of negatively charged functional groups onto the surface of the SMP would specifically aid in adsorption of cationic EDGPs.

The purpose of this study was to design a shape memory polymeric surface to promote protein adsorption for the eventual binding of specific biomarkers of EoE. The results of the study show significant amounts of protein adsorption, specifically bovine serum albumin (“BSA”), onto the surface of the SMP’s after the incorporation of negatively charged sulfonic acid groups coupled with extensive adsorption system adjustments.
2. Background

2.1 Eosinophilic Esophagitis and the Esophageal String Test

Eosinophilic esophagitis is an emerging chronic inflammatory disease of the esophagus effecting thousands of Americans each year\textsuperscript{10}. The disease is characterized by the infiltration of eosinophils to the esophagus. These cells are a gateway to esophageal inflammation as they have the capacity to initiate an inflammatory cascade causing a diverse set of symptoms. Serious complications include food impaction and stricture formation that could require urgent removal of the food or endoscopic balloon dilatations\textsuperscript{10}. The current requirement for diagnosing this disease is through esophagogastroduodenoscopy ("EGD") and histological examination of esophageal mucosal biopsies\textsuperscript{10}. These methods can be both inaccurate and unreliable. Biopsies represent 0.7\% of the esophageal surface area, a small enough sample size to miss the disease\textsuperscript{3}. Endoscopies depend on abnormal physical features of the lumen that may not be present in all diseased patients\textsuperscript{2}.

The Enterotest, performed originally to detect parasites in the intestine, is under experimentation for capturing highly cationic EDGPs, biomarkers of EoE in the mucosa of the esophagus\textsuperscript{4}. This esophageal string test is a nylon string that is packed into a capsule. During the procedure, one end of the string is taped to the cheek and the remaining string is carried through the GI tract, unraveling from the weighted capsule upon swallowing. The capsule is dislodged from the string and mucosal remnants are captured onto the material. Further \textit{in-vitro} detection of EDGPs is performed via western blots, ELISA’s and other antibody driven tests. Although this is a great advancement in a noninvasive diagnostic for EoE, there is room for improvement. In our application, we are altering the surface of the SMP to capture these cationic biomarkers of EoE in a similar fashion to the Enterotest. The shape memory effect of the polymer will aid in positioning the functionalized surface near the site of the biomarkers while the modified surface is designed to specifically adsorb the EDGPs of EoE. This can improve diagnosis considerably by obtaining greater sampling sizes and capturing specific proteins of the disease.
2.2 Adsorption Principles

Before the surface was modified, the principles of adsorption were defined and understood. Adsorption is the process of particulate or molecular binding onto a surface. Surfaces are not fully bound and consumed by their surrounding atoms as their bulk material; they have the capacity for binding atoms, an energy favorable mechanism that lowers their energy state. Thus, surfaces usually possess higher surface energy than their bulk material which we might identify with as a good adsorbent.8

Since the surfaces of polymers usually have low surface energy and are not as reactive as other surfaces such as metal because of their low chemical potential, functional groups were incorporated to facilitate pro-adsorption characteristics for EDGPs11. Protein adsorption is a complex mechanism primarily because of the myriad of influential factors it encompasses, surface energy being one of them. It’s a function of the polymer’s surface, the protein being adsorbed, and the solution they are in. All of these entities have their own list of influencing characteristics displayed in figure 1. The ones that overlap with each other were heavily implemented in the final experimental design.
The stages that comprise protein adsorption onto a polymeric surface are: 1- the transport of protein near the surface, 2- the attachment of protein onto the polymeric surface, 3- the rearrangement and reorientation of the protein onto the surface, and 4- the desorption or permanent attachment of protein onto the polymeric surface. The last stage is dependent on the extent of relaxation of the protein upon binding. As the protein contacts the surface, interaction forces increase, and if large enough to compete with the protein’s intermolecular forces, the protein will start to denature, encouraging irreversible binding. If the residency time is maximal, the protein will have sufficient time to relax before desorption permits it.

Minimal residency time is due to few and weak interaction forces upon initial binding or the interference with other competing proteins.

In the first step of adsorption, the transport of protein near the surface of the polymer is due mostly to coulombs interactions (electrostatic interactions) with an interaction force of $\frac{1}{r^2}$ where $r$ is the distance of
the protein from the polymer’s surface. As the protein is drawn closer, Vander Waals forces, with an interaction force of \( \frac{1}{r^6} \), persists as the driving force for the initial stages of adsorption\(^{12}\).

The second stage, protein attachment, occurs if the polymer elicits active sites that compliment the amino acids on the surface of the protein. Particularly functional groups such as COOH, OH, NH2, SH, SO3 and hydrophobic residues on both the surfaces of polymers and proteins, aid in the initial attachment onto a polymeric biomaterial. Though this usually consists of multiple weak interactions, if more time is allowed, they become stronger and irreversible which affect the third stage, reorientation and relaxation onto the polymeric surface\(^{8,12,13}\).

The influential factors in figure 1 can determine if the protein will remain bound irreversibly or bind momentarily and desorb back into solution, the 4\(^{th}\) stage in protein adsorption. If the adsorbent competes with the intermolecular forces that keep the protein structurally sound, the protein is most likely to bind irreversibly due to permanent conformational changes. For irreversible binding to occur, the protein must unveil its hydrophobic core to the adsorbent. The more heterogeneous the intermolecular forces are on the adsorbent, the more likely it will be able to unfold the protein and permit maximal residency time for irreversible binding. It is for this reason, the SMP was modified to have a negative charge and accrue the EDGP\(_s\) to its surface, encouraging interaction with both electrostatic and hydrophobic chemical constituents.

### 2.3 SMPs and Surface Modification

Biological responses can be reprogrammed from the surface of biomaterials rendering polymeric surfaces important considerations to biomedical applications\(^{14}\). Surface texture, surface potential and surface energy are mainstream surface features that can influence aggregation and/or binding to the surface and trigger such host response\(^{15}\). Particularly, surface chemistry and topography dominate biological responses to biomaterials\(^{16}\). By altering the surface profile, biological responses can be manipulated specifically to adsorb protein onto the surface, an important consideration in the field of biomedical applications\(^{8}\). Concomitantly, bulk polymeric properties attribute to the core mechanical strength and agility of the device, playing an integral role in establishing durability, flexibility and comfort. SMPs extend this role much further as they are a class of polymeric materials with the ability to hold a ‘fixed’
temporary state and recover a ‘memorized’ permanent state upon the introduction of an external stimulus 17–19. In our application, the SMP was utilized for the base component for this device because of this intrinsic ability to memorize a predefined shape. An acrylate based SMP was used, with the ability to be thermally stimulated to initiate shape transitions. Because the SMP can be stored in a temporary fixed state for elongated periods of time, it can be packed and stored in a small capsule. Upon the introduction of the polymer to the esophagus, the SMP is thermally stimulated and has the ability to undergo large recoverable deformations. This characteristic can be easily tailored such that the polymer returns to a site near the biomarkers and increases its surface area substantially. Together, these properties encourage adsorption by increasing the loading capacity of the polymer and placing them in close proximity to the biomarkers to shorten their transport path. The union of SMPs and refined surface properties can pave the way for a myriad of biomedical devices. For this study, the focus is on designing an optimal SMP surface to promote binding of unique cationic, EoE biomarkers for the development and improvement of a diagnostic for this disease.

The base formulation of the SMP is mostly hydrophobic, an encouraging factor of protein adsorption in general. Incorporation of negatively charged groups onto the surface can facilitate specific and preferential binding to eosinophil derived granule proteins: major basic protein 1 ("MBP1"), eosinophil cationic protein ("ECP"), eosinophil derived neurotoxin ("EDN"), and eosinophil peroxidase ("EPX").
3. Rational for the Experimental Design and Important Considerations for Adsorption Studies

3.1 Prelude to Rationale

In this study, the initial aim was to modify the surface of an acrylate-based SMP to adsorb specific cationic proteins of EoE. Because protein adsorption is a complex mechanism, the initial experiments for this goal yielded no binding. System adjustments were iterated throughout a major portion of this study with a continued failure to adsorb proteins onto the SMP surface. The goals of the study broadened to modifying the surface of the SMP to eventually adsorb cationic proteins. Until the core principles of protein adsorption were understood, the specific adsorption of cationic proteins would be difficult to meet. A commercially available adsorbent that served as a positive control was issued in the experimental process to check against previous experiments and their environments. This analysis allowed us to see what particular factors are encouraging or discouraging for protein adsorption. From this, the degree of complexity of adsorption was revealed as multiple factors can significantly inhibit or attenuate binding. After final system revisions were made from the positive control findings, significant amounts of BSA were adsorbed onto the modified polymer surface. Because of the numerous system revisions that were implemented in this study, detailed below is the rationale for considering these important factors that contribute to the multifaceted mechanism of adsorption.

3.2 Polymer

3.2.1 Major Interactions Forces Involved in Adsorption

Electrostatic, hydrophobic and hydrogen bonds are cited as the major interaction forces in protein adsorption\textsuperscript{5,7,8,12}. However, the dominating factor is still under controversy, as the literature remains inconsistent due to the complexity of adsorption. As such, all three interaction forces were studied. The base formulation of our SMP is mostly hydrophobic, providing one of the major interaction forces. Tert-Butyl Acrylate (“tBA”), the hydrophobic monomer in the SMP formula is displaced by the weight percent of the new functional monomer being incorporated into the mixture, minimally decreasing the hydrophobicity but increasing the other interaction forces.

For the initial goal of adsorbing cationic protein onto the surface of the polymer, negative electrostatic interactions were implemented. Methacrylic acid (“MA”), 2-carboxyethyl acrylate (“CEA”) and 2-sulfoethyl methacrylate (“SEM”) were chosen as monomers that would exhibit an electrostatic
charge under physiological pH and were incorporated into the SMP at various weight percents. Below is a schematic of how the polymer surface would exhibit key interaction forces of adsorption. At pH 7, the hydrogen on the carboxylic acid of MA and CEA would disassociate and leave the oxygen species negatively charged. The negatively charge surface of the polymer can provide interaction sites for the cationic proteins, encouraging adsorption through both electrostatic and hydrophobic (Van der Waals and London type) interactions.

Figure 2  Schematic of Major Adsorption Interaction $^{6,7}$

Methacrylic acid, 2-carboxyethyl acrylate and 2-sulfoethyl methacrylate was incorporated into the SMP at increasing weight percentages to facilitate major interaction forces of adsorption.

Because the incorporation of MA and CEA at pH 7 did not yield protein binding, the system was adjusted such that the pH matched the isoelectric point of the study protein. At the new pH (4.5), the hydrogen atom on the carboxylic acid group should not disassociate, thus leaving the hydrogen intact to facilitate hydrogen bonding.

After these polymeric alterations, binding still did not persist. The last modification was made with SEM. We proposed this new monomer would work because it was a stronger acid than carboxylic acids used previously and may possess a larger interaction force for protein capture. Literature also verified using SO3 functional groups for protein adsorption$^{5}$. 
3.2.2 Shape Memory Polymer

An acrylate based SMP was employed as the base polymer mainly because of its ability to self deploy in the esophagus near the site of the EoE biomarkers and its high specific surface area after shape recovery.

Additional benefits are its ability to be packed into a small capsule in its temporary state for an elongated period of time. The SMP is also easily processed, cost efficient, non invasive and has great mechanical properties.

3.2.3 Surface Area

Surface area directly correlates to the loading capacity of the polymer for protein. If the surface area is minimal, small samples sizes are retrieved, and make detection difficult. As such, a course of action was taken to increase surface area substantially and remained a focus for many experiments.

3.2.4 Impurities on the Polymeric Surface

Careful processing of the SMP is crucial for binding experiments as the surface is highly reactive and contamination is fairly easy. The polymer samples were handled with gloves and under clean conditions. The samples were always stored in a Ziploc bag, away from air particulates, before the binding experiments.

During the initial experiments, the shape memory coupons were washed with Sparkleen. Because this is an amphiphilic molecule, one concern was the hydrophobic portion of the molecule binding irreversibly to the polymer surface. Even if the bond was reversible, impurities such as this become part of the adsorption system and could interfere with binding by taking up sites on the protein or polymer that shield the interaction forces we were counting on and altering the adsorption kinetics. To prevent occupancy of the binding sites with such impurities, the polymers were switched to being washed with clean nano pure or distilled (DI) water. The water source is important as well because organic and ionic species can bind to the polymer surface, altering the charge or taking vacancy on limited binding sites. All surfaces and equipment the polymeric samples came into contact with were wiped down with methanol or ethanol and DI water.
3.2.5 Post Processing

Initially, all polymers were post cured for 1 hour at 90°C. Since binding did not occur, one explanation was the possibility of free monomer that was not evaporated during the post cure. As mentioned above, these impurities can take up valuable binding sites on the protein and polymer while shielding important interaction forces. Thus, methanol treatments were carried out to swell the polymer and eliminate leachable content. However, methanol treatments were swelling the polymer to approximately 1/3 of its own size, which ultimately could irreversibly expand the pore sizes of the polymer networks and influence adsorption. Since our goal was to adsorb protein through electrostatic charge, the altered pore size added an additional avenue of protein adsorption. Additionally, when methanol treatments were carried out with CEA at 5 and 15wt%, the samples cracked after vacuum drying them for 48 hours. In effect, methanol treatments were stopped.
48 hour vacuum drying and methanol treatments on 5 and 15 wt% CEA causes polymeric cracks.

Sterilization was examined to ensure riddance of impurities. Polystyrene and SEM samples (0, 0.5, 1 and 2.5 wt %) were placed in an autoclave after DI washing. The 2.5 and 1% SEM samples did not survive this process as they encountered numerous cracks. Additionally, the polystyrene samples became distorted so we reverted back to the original post cure of 1 hour at 90°C.
Polymeric breaks and distortion were created from autoclaving samples at 250°F for 15 minutes. The first box shows 0, 2.5 and 0.5% SEM SMPs while the second encompasses polystyrene and 1% SEM SMP samples.

3.3 Protein

3.3.1 General Considerations for the Study Protein

The structural mobility, compactness, size, intramolecular forces and charge were all considered in picking a study protein. High structural mobility encourages irreversible binding onto the polymer surface by readily making conformational changes upon adsorption. This mobility allows for increased contact numbers onto the polymeric surface and thus heightens the affinity of the protein for the polymer. Internal mobility also increases the rate of binding.

The intramolecular forces maintain structurally stability of the protein; hence, less internal motion occurs. Therefore, these intramolecular forces are what the adsorbent is competing against for final conformational changes to take place that lead to irreversible binding. If the intramolecular forces are strong and plentiful, more energy is required from the adsorbent to denature the protein. Therefore, less intramolecular forces are better for this application. The number of disulfide bonds and apolar groups are
key contributors to the proteins stability. Apolar groups also contribute to the compactness of the protein, an anti-adsorption variable.

The size of the protein is directly correlated to the number of interaction forces it can make. Since a larger protein can make more contacts onto the polymer surface, the strength of the total adsorption bond is stronger and will trend toward irreversible binding. A charged protein is structurally loose because of intramolecular repulsion between the charged residues. This provides extra molecular mobility. Since the target proteins are EDGPs, a positively charged analog was used. Below are the proteins picked for this study and why.

Table 1 illustrates major protein characteristics that influence protein adsorption.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>% Hydrophobicity</th>
<th>% Charged Sites</th>
<th># disulfide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly L Arginine</td>
<td>5-15</td>
<td>10.76</td>
<td>------</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>ECP</td>
<td>18.39</td>
<td>11.4</td>
<td>43.16</td>
<td>20.63</td>
<td>9</td>
</tr>
<tr>
<td>EDN</td>
<td>18.35</td>
<td>8.9</td>
<td>41.62</td>
<td>15.53</td>
<td>9</td>
</tr>
<tr>
<td>EPX</td>
<td>81.04</td>
<td>10.8</td>
<td>43.24</td>
<td>24.2</td>
<td>17</td>
</tr>
<tr>
<td>MBP</td>
<td>25.21</td>
<td>10.9</td>
<td>40.54</td>
<td>26.12</td>
<td>12</td>
</tr>
<tr>
<td>BSA</td>
<td>69.29</td>
<td>4.7</td>
<td>42.83</td>
<td>33.27</td>
<td>35</td>
</tr>
</tbody>
</table>

3.3.2 Poly-L-Arginine

Poly-L-arginine was chosen because it is a cationic protein with a similar isoelectric point (10.76) to the EDGPs.

3.3.3 Eosinophil Derived Granule Proteins

Poly-L-arginine did not bind to the negatively charged polymeric surface. Since these synthetic analogs did not have hydrophobic domains to make irreversible interactions, EDGP’s were used in place. These are the actual target proteins for the diagnosis and will provide the correct intramolecular forces that we will be competing against. They were also chosen for the study protein after poly-L-arginine because of the high density of interaction forces these native proteins intrinsically have on their surface and core to promote irreversible binding.
3.3.4 Bovine Serum Albumin

BSA was primarily used because it is widely characterized and was the main study protein in other adsorption studies.

Careful consideration of the experiments was taken because of the proteins tendency to oligomerize over time or during elevated temperatures. Because oligomerization creates a bigger molecule, it may adsorb more readily (quickly and firmly) than single BSA molecules. BSA also possesses pro-adsorption characteristics because of its 1) high structural mobility, 2) hydrophobic cleft on its surface and 3) moderate size for more interactions (larger than most EDGP’s).

3.4 Solution

3.4.1 Solution’s Importance

The solution is especially important in our experiments as it controls the ionization states of both the protein and polymeric surfaces. In essence, the negative, electrostatic interactions incorporated on the polymeric surface are dependent on the pH and ionic strength of the solution. Because the negative charges on the SMP specifically serve to bind the target, cationic proteins, it illustrates how critical the solution is to EDGP adsorption. Similarly, if the pH and competing ions in solution displace the cationic charge on the protein’s surface, the specificity of the SMP device is eliminated.

Initially, solutions mimicking physiological parameters were implemented but because of the high ionic strength, amino acid content and other interfering components, other buffers were considered. After the process of elimination, ionic strength and the pH of the solution deemed the most important factors to consider and were adjusted appropriately.

3.4.2 Temperature

All the adsorption studies were executed at physiological temperature (37°C). Increased temperatures accelerate the transport of the protein near the interfacial region of the polymer surface and contribute to internal mobility of the protein.
3.4.3 pH

The pH influences charged groups onto the surface of the polymer and protein. If the pH is under the isoelectric point of the species, it acquires a net positive charge. If the pH is above, the species has a net negative charge and if the pH is at the PI of the species, it is neutral. This is a central component to protein adsorption as it can impose repulsion forces between proteins and can change the acid/base nature of the polymer surface.

3.4.4 Ionic Strength

Ionic strength is crucial to protein adsorption as it defines the degree of interfering counter ions in solution. These ions can shield the charge that was designed for EDGP attachment or consume binding sites on the protein itself. In this way, ions have the strength to weaken or persuade protein adsorption one way or the other. The final experiments were performed in low ionic strength conditions (0.01M).

3.4.5 Purity

The cleanest reagents, including water, must be used. Ions and organic species compete with the target proteins to adsorb onto the polymer surface. Not only do they consume valuable binding sites in lieu of the targeted protein but also they can alter the charges of both the protein and polymer in which the SMP device is reliant on for specific EDGP adsorption.

DI water was passed through a 0.2um filter and used to make up all solutions. All the beakers and materials that came into contact with the adsorbents were autoclaved or sterilized by methanol/ethanol prior to use.
4. Materials and Methods

4.1 Materials

Tert-butyl acrylate monomer, poly(ethylene glycol) dimethacrylate (“PEGDMA”) (Mn=550) crosslinker, CEA monomer (552348-50ML), MA monomer and the photo initiator 2, 2-dimethoxy-2-phenylacetophenone (“DMPA”) were ordered from Sigma Aldrich. SEM monomer was ordered from polysciences (cat 02597-50). Aliquots of poly-L-glutamic acid (Sigma Aldrich MW 3,000-15,000, Stock p4636-25mg) and poly-L-arginine (Sigma Aldrich MW 5,000-1500, Stock P4663) were prepared at 2,500ug/ml in PBS (Invitrogen) diluent. Nylon (ASTM D4066 PA-0114, White Amazon Supply) and polystyrene sheets (McMaster-Carr 8734K39 1/16” Thick) were purchased to examine their adsorbent potential compared to SMPs. Nylon is the composite material of the esophageal string test and polystyrene is a known adsorbent that was utilized as a positive control. ECP and ECP ELISA’s were purchased from MBL International Corporation. The Micro BCA Protein Assay Kit (Thermo Scientific / Pierce 23235) was the primary protein detection method.

4.2 Methods

4.2.1 Characterization

4.2.1.1 Contact Angle and Surface Free Energy

Polymer surfaces were examined through a goniometer. A flat polymer was placed on a stage and a volume of DI water or Diiodomethane (Sigma-Aldrich 158429-25G) was slowly dropped onto the surface. The droplet was photo documented and the angle between the interfacial solid and liquid was calculated through the automated software. The surface energies were calculated through Fowkes equation depicted below. The surface energies of the associated liquids are listed as well. Note: Because surfaces are active species, precaution was taken in handling and processing them to avoid contamination or destructs within its smooth surface.
Fowkes Equation\(^{12,20,21}\):

\[
\gamma_s = \gamma^d + \gamma^p
\]

\[
\gamma_t = \gamma^d + \gamma^p
\]

\[
\gamma_s = \gamma_s^d = 0.25\gamma_t(1 + \cos\theta)^2
\]

\[
\gamma_s^p = \frac{0.5 \gamma_t(1 + \cos\theta) - (\gamma_s^d \gamma_t^p)^{0.5}}{\gamma_t^p}
\]

\[
H2O = \gamma_t^d = 21.8 mJ/m^2
\]

\[
H2O = \gamma_t^p = 51.0 mJ/m^2
\]

\[
Diyiodomethane = \gamma_t^d = \gamma_t = 50.8 mJ/m^2
\]

4.2.1.2 pH Testing

12x59x1mm CEA SMP samples were placed in 10mL of Acetate buffer at a pH of 4.45. Stir bars were added to each sample and were placed on a stir plate. The pH was recorded every hour for 4 hours. The experiment was performed in duplicates.

4.2.1.3 Water Equivalency Test

SEM polymers, nylon and polystyrene were dehydrated in a vacuum oven for 48 hours at 60 C\(^0\) and weighed for a baseline measurement. The polymers were incubated in excess DI water at 37C. The polymers were weighed and recorded every 12 hours until they reached equilibrium, at 24hours. The experiments were performed in duplicate.

4.2.1.4 Dynamic Mechanical Analysis

All CEA and SEM SMP samples were sized to 5x30x1mm and their edges were sanded with 600-grit sand paper. The samples were cycled at 0.1HZ, with a heating rate of 3C\(^0\)/min with the testing temperature ranging from 0-100C\(^0\). The glass transition temperature ("Tg") was determined by the peak of the tan delta curves.
4.2.1.5 Fourier Transform Infrared Analysis

All CEA and SEM samples were polymerized between two glass slides with a thin spacer < 0.5mm. Free radical polymerization was initiated and propagated with a UV source (black ray) at an intensity of ~11mW/cm$^2$ for 30 minutes. The samples were carefully removed and heat treated for 1 hour at 90°C. Fourier transform infrared spectroscopy (“FTIR”) spectra were taken and the disappearance of the alkenes peak at 1610-1680cm$^{-1}$ was used to determined convergence.

4.2.2 Adsorption Testing

4.2.2.1 SMP Synthesis

SMPs were synthesized using tBA monomer, PEGDMA crosslinker and DMPA photoinitiator. MA, CEA or SEM monomers were added to the base formulation listed initially at different mass fractions displacing tBA fractions. Solutions were made by mixing desirable weight percentages of monomers, tBA, PEGDMA and 0.1wt% of DMPA in a glass vial. The solutions were injected into a pre-casted mold made from two glass slides separated with 1mm spacers. A UV lamp (Black-Ray) was used to polymerize the solutions at an intensity of ~11mW/cm$^2$ for 30 minutes. After polymerization, the polymer coupons were removed from its cast and were heat treated at 90°C for 1 hour to evaporate unreacted monomers. The samples were sized and their edges sanded with 600 grit sand paper to even out texture introduced from sizing. Afterwards, the samples were methodically washed with DI water.

4.2.2.2 Binding Experiments

SMP samples were incubated in protein solution for 1 hour at 37°C. A protein only condition was incubated alone for quantification of initial protein concentrations. Media only (no SMP added) and SMP incubated in the media (no protein) are additional conditions that were used as background controls. All experimental conditions were performed in triplicate. Supernatant was collected in separate eppendorfs and frozen in a -20°C freezer until further use. Either the solution depletion method was used to quantitate protein bound indirectly to the samples or an extra elution step was performed to quantitate protein bound directly. With the elution step, the polymer sample was rinsed with PBS and dabbed with a Kim wipe before incubation with EST buffer. The samples were incubated for 30 minutes with a 30 second vortex step after ten minute intervals. The free solution was collected and frozen until further use. An alternative is
the solution depletion method where the concentration of free protein after incubation with the SMP sample was subtracted from initial protein concentration.

*Any deviations from these methods are noted in the experiment itself below.

4.2.2.3 **Experiment 1. MA**

SMP coupons were prepared with 0, 5 and 15 wt% of MA (0:80:20, 5:75:20, 15:65:20 [MA: tBA: PEGDMA]). A UV source was used for polymerization with the intensity ~20mW/cm². Circular discs were formed with a 6mm dye (McMaster-Carr 3418A6). The discs were cleaned with methanol and DI water several times and dried at 90°C for 1 hour. Samples were parafilmed in glass beakers overnight and used the next day for the binding experiment.

SMP discs were incubated in 44ug/ml of poly-L-arginine and poly-L-glutamic acid for 5 minutes, 30 minutes and 2 hours at 37°C in 24 well plates. PBS was used because of its compatibility with the BCA assay and the surface to volume ratio was 37.68mm². The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.

4.2.2.4 **Experiment 2-3. CEA**

SMP coupons were prepared with 0, 5 and 15 wt% of CEA (0:80:20, 5:75:20, 15:65:20 [CEA: tBA: PEGDMA]). A UV source was used for polymerization with the intensity ~20mW/cm². Circular discs were formed with a 10mm dye (McMaster-Carr 3418A1). The discs were cleaned with Sparkleen and DI water several times and left out to dry overnight. Nylon was sized, sanded, washed and dried in the same manner as the SMPs for experiment 3.

SMP discs were incubated in 44ug/ml of poly-L-arginine and poly-L-glutamic acid for 1 hour at 37°C in 24 well plates. PBS was used because of its compatibility with the BCA assay and the surface to volume ratio was 94.2mm². Six experimental repeats were performed per condition to help decrease the error bars. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.
4.2.2.5 Experiment 4. Surface Area 132mm$^2$/ml

SMP coupons were prepared from the base formulation 80:20 (tBA: PEGDMA). A UV source was used for polymerization with the intensity ~20mW/cm$^2$. Square samples were formed with the dimensions of 20x20mm. The samples were cleaned with Sparkleen and DI water several times and left out to dry overnight. Nylon was sized, sanded, washed and dried in the same manner as the SMPs for experiment 3.

SMP discs were incubated in 44ug/ml of poly-L-arginine for 1 hour at 37$^\circ$C in glass beakers. PBS was used because of its compatibility with the BCA assay. Three square samples were added per condition. Because of the increased surface area, more protein solution had to be added (20mL) to cover the surfaces. As such, the surface to volume ratio was 132mm$^2$/ml. There were no experimental replicates because of the large polymer samples. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.

4.2.2.6 Experiment 5. Surface Area with SMP Particles

SMP coupons were prepared from the base formulation (80:20 tBA: PEGDMA) in. A UV source was used for polymerization with the intensity ~20mW/cm$^2$. The coupons were cleaned with Sparkleen and DI water several times and left out to dry overnight. Polymer and nylon particles were created from a dremel and collected into a vacuum device depicted below. The samples were imaged using an optical microscope at 6X magnification to see the range of sizes created. Small particles were disposed of through centrifugation steps (particles were passed through a 230um sieve to rid small particles but this did not work well) so as not to interfere with the BSA assay and elicit a false positive result. One gram of SMP particles were measured in a polypropylene 50mL conical. Ultra pure H20 was added to the conical and spun at 1325 RCF at RT for 10minutes. The smaller particles were found on top and were decanted. Three of these washes were performed as displayed below. The particles were vacuum dried for 2.5 hours. SMP particles were weighed at 0.08, 0.04, 0.02, 0.01, 0.005 and 0.0025 grams and added to an ultra low attachment (“ULA”) 6well plate. To determine the maximal surface to volume ratio, 1mL volumes of PBS were added to each sample until 8mL was reached per well. With the findings, 0.06 and 0.04g were used for the binding experiment.
A. Vacuum System to Collect Polymer Particles

Use a dremel to create polymer particles. This is carried out over the funnel for collection.

Polymer Particles are fed into the 50mL eppendorf tube.

Tube feeds to Vacuum outlet.

B. SMP Particle wash step

Wash 1

Wash 2

Wash 3
The particles were weighed at 0.04 and 0.06 grams and added to a ULA 6 well plate (Costar Product 3471). 5mL of 44ug/ml of poly-L-arginine was added and the samples were incubated for 1 hour at 37°C in a shaker at 115RMP. PBS was used because of its compatibility with the BCA assay. After the incubations, 1mL of supernatant was aspirated from each condition into separate eppendorfs and spun at 13,000 g for 10 minutes at RT. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.

4.2.2.7 Experiment 6. Surface Area with SMP Particles - Complete Submersion

Refer to Experiment 5. 1.22 grams of SMP particles were weighed and placed in glass cryovials. 1.7mL of 44ug/ml of poly-L-arginine was added and the samples were incubated for 1 hour at 37°C in a shaker at 200RMP. PBS was used because of its compatibility with the BCA assay. After the incubations, 1mL of supernatant was aspirated from each condition into separate eppendorfs and spun at 13,000 g for 10 minutes at RT. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.
4.2.2.8 Experiment 7. Surface Area 648mm$^2$/ml

SMP coupons were prepared from the base formulation 80:20 (tBA: PEGDMA). A UV source was used for polymerization with the intensity ~20mW/cm$^2$. Square samples were sized to 20x20mm and cut further into ~5x6mm samples. The samples were cleaned with Sparkleen and DI water several times and left out to dry overnight. Nylon was sized, sanded, washed and dried in the same manner as the SMPs. Next, the samples were incubated in methanol for 48 hours to alleviate competing leachables that may still be present after post heat treatments. Afterwards the samples were washed with DI water and dried in a vacuum oven at 60°C for 48 hours.

SMP pieces were incubated in 44ug/ml of poly-L-arginine for 1 hour at 37°C in glass vials. PBS was used because of its compatibility with the BCA assay. Three 20x20mm square samples each cut into 5X6mm samples were added per condition. 5mL of protein solution was added per condition for a final surface to volume ratio of ~648mm$^2$/ml. The samples were incubated at 37°C and shaking at 200RPMs. The samples were specifically oriented upright to limit them from floating to the top. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.

Figure 7 Adsorption Optimization Techniques

Orient the vials so they are standing up to limit polymers from floating to the top

4.2.2.9 Experiment 8. Eosinophil Lysate

SMP coupons were prepared from the base formulation 80:20 (tBA: PEGDMA). A UV source was used for polymerization with the intensity ~20mW/cm$^2$. Coupons were washed with Sparkleen and DI water and dried at room temperature. The coupons were cut into ~6X30mm wide strips and submerged in
methanol for 48 hours. The strips were vacuum dried for 48 hours at 60°C. Afterwards samples were sized to 5X12mm and their edges were sanded with 600-grit sand paper. Next, the samples were washed with DI water and dried once more for 1 hour at 60°C in a vacuum oven. Samples were stored in a Ziploc bag until further use. Nylon was treated in the same throughout all experiments. It was cleaned, cut and sized in the same manner as the SMP samples. No post treatments were necessary except for 1 hour incubation at 90°C after it was washed.

Eosinophils ("EOS") were isolated from peripheral blood. The cells were suspended in 0.025M of sodium acetate buffer (pH of 4.3) with 10% protease inhibitor (Roche) at a concentration of 5x10^6 cells/ml. The cells were sonicated to create EO lysate and spun down at 300g for 10 minutes. The supernatant was collected and frozen at -80°C until use.

SMP samples were incubated in 500k/ml of blood eosinophil lysate per RPMI + 8% FBS buffer for 1 hour at 37°C, final pH 7. The surface to volume ratio was 154mm^2/ml. An elution step was carried out after the binding experiment. The supernatant was collected and ECP, EDN and MBP1 ELISAs were performed.

**4.2.2.10 Experiment 9-10. Eosinophil Lysate**

The experimental parameters remained the same as experiment 8. The only exception is the polymer samples did not undergo methanol treatments. The polymer samples were cleaned with Sparkleen and DI water. After this washing step, the polymer was vacuum dried at 90°C for 3.5 hours. The coupon was cut, sized to 5x12mm samples and sanded. Next, they were washed with DI water and heat treated once more for 1 hour at 60°C in a vacuum oven to ensure the riddance of water. They samples were placed in a Ziploc bag for storage.

**4.2.2.11 Experiment 11. Eosinophil Lysate**

To compare heat treated to methanol treated polymers, both post processing techniques were administered. See experiment 8 and 9-10 for polymer preparation.

All parameters stayed the same with the EO prep except the low 300g spin was switched to a high speed spin at 13,000g to ensure not sonicated cellular debris was pulled down and excluded from the lysate for binding.
4.2.2.12 Experiment 12. CEA

SMP coupons were prepared with 0, 5 and 15 wt% of CEA (0:80:20, 5:75:20, 15:65:20 [CEA: tBA: PEGDMA]). After the standard polymerization and heat treatment, the samples were washed with Sparkleen and DI water. SMPs were sized to 5X12mm dimensions and their edges were sanded with 600-grit sand paper. Finally, samples were washed with DI water several times and dried at 90°C for one hour.

SMP samples were incubated in 1mg/ml of BSA (Sigma) for 3 hours at 37°C in 1.5mL eppendorf tubes. Acetate buffer (40% of 0.1M acetic acid + 60% 0.1M sodium acetate) was used at a pH of 4.5. The surface to volume ratio was 15mm²/ml. Three experimental repeats were performed per condition. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.

4.2.3 Positive Control- Optimization of Polystyrene Microspheres

4.2.3.1 General procedure

Polystyrene microspheres were purchased from Bang’s Laboratories, Inc (Catalog code DS03V, 0.51um). The polystyrene microspheres were initially optimized for the appropriate quantity-to-surfactant ratio with respect to adsorption. Afterwards adsorption was tested against protein concentration, buffer solution, reproducibility and surfactant-less solution. All the experimental groups remained the same from the previous binding experiments: protein only, media only (no microspheres added), microspheres incubated in media (no protein) and microspheres incubated with protein. The groups were performed in triplicate. The samples were incubated in 120ug/ml for 3 hours at 37°C unless indicated otherwise. After the incubation, the microspheres were spun at 9300 G for 15minutes at 10°C. The supernatant was carefully collected in a separate eppendorf tube and spun again at 9300G for 15min at 10°C. The supernatant was collected and the BCA assay was run. The solution depletion method was used for quantification.
4.2.3.2 Surface Area and Surfactant Optimization

Microspheres were diluted 1:10, 1:100 and 1:1000 in acetate buffer (pH 4.45, Ionic strength 0.01M, reagent grade materials used with filtered DI H2O) and incubated in BSA with a final concentration of 120ug/ml for 3 hours at 37°C.

*First 1:10 dilution was made by adding 300ul of microspheres into 2,700ul of acetate buffer

4.2.3.3 Total Exclusion of Surfactants

40ul of microspheres were placed in a centrifuge tube and diluted with acetate buffer. The tube was vortexed to wash the microspheres and spun at 14,000 g for 5 minutes at RT. The supernatant was discarded and one more wash was performed on the spheres. Next, the microspheres were diluted 1:100 in acetate buffer (pH 4.45, Ionic strength 0.01M, reagent grade materials used with filtered DI H2O) and incubated in BSA with a final concentration of 120ug/ml for 3 hours at 37°C.

4.2.3.4 BSA Concentration Optimization

The microspheres were diluted 1:100 in acetate buffer (pH 4.45, Ionic strength 0.01M, reagent grade materials used with nano-pure H2O) and incubated in BSA with a final concentration of 120ug/ml or 40ug/ml for 3 hours at 37°C.

4.2.3.5 Buffer Solution Optimization

The microspheres were diluted 1:100 in either acetate buffer made from nano pure water or DI water passed through a 0.2um filter. The diluted spheres were incubated in BSA with a final volume of 120ug/ml for 3 hours at 37°C.

4.2.3.6 Reproducibility Testing

The reproducibility of adsorption with polystyrene microspheres was tested with pipetting small versus large quantities of the microspheres when the microsphere dilutions were prepared. The microspheres were diluted 1:100 by two 1:10 serial dilutions. The first 1:10 dilution determined the error as the second dilution stayed the same for both conditions. The “small” quantity was prepared by adding 40ul of microspheres into 360ul of acetate buffer (pH 4.45, Ionic strength 0.01M, reagent grade materials used with filtered DI H2O) for the first 1:10 dilution, the “large” quantity was prepared by adding 300ul into
2700μl of acetate buffer for its first 1:10 dilution. The second dilution remained the same for both conditions; a 1:10 was performed by adding 300μl of the already prepared 1:10 dilution into 2700μl of acetate buffer. The final diluted microspheres (1:100) were incubated in BSA with a final concentration of 120μg/ml for 3 hours at 37°C.

4.2.3.7 BSA Alexa Fluor 488

Polystyrene microspheres were diluted 1:100 in acetate buffer (pH 4.45, Ionic strength 0.01M, reagent grade materials used with filtered DI H2O) and incubated in BSA alexa fluor 488 (Invitrogen A13100) at a final concentration of 120μg/ml for 3 hours at 37°C.

4.2.4 Positive Control – Test Polystyrene Microspheres against Past Experiments

4.2.4.1 General Procedure

The polystyrene microspheres were treated the same as before. The microspheres were diluted 1:100 in the appropriate buffer specified and incubated with the study protein. The microspheres were spun at 9300 G for 15 minutes at 10°C. The supernatant was carefully collected in a separate eppendorf tube and spun again at 9300G for 15 min at 10°C. The supernatant was collected and the BCA assay was run. The solution depletion method was used for quantification.

4.2.4.2 Experiments 1-7 Poly-L-Arginine

Polystyrene microspheres were diluted 1:100 in PBS and incubated in 44μg/ml of poly-L-arginine for 30 minutes, 1 hour, 2 hours and 3 hours at 37°C.

4.2.4.3 Experiments 8-11 Eosinophil Lysate

Polystyrene microspheres were diluted 1:100 in RPMI with 8% FBS and incubated in 500k of EO lysate (lysate was suspended in 0.25M sodium acetate buffer + 10% PI) for 1 hour at 37°C.

4.2.4.4 Experiment 12 BSA and Ionic strength

Polystyrene microspheres were diluted 1:100 in either high ionic strength acetate buffer (0.1M) or low ionic strength acetate buffer (0.01M). The spheres were incubated in BSA at a final concentration of
1mg/ml for the high ionic strength condition and 120ug/ml for the low ionic strength condition. The samples were incubated for 3 hours at 37°C.

4.2.5 SEM Adsorption

SEM polymers were synthesized by free radical polymerization using 0.4 wt% of DMPA photo initiator. Mixtures of SEM monomer, the PEGDMA crosslinker and tBA monomer were injected between the glass mold mentioned previously with the exception of a 1.5mm spacer. Polymerization was performed under a UV lamp source with an intensity of ~11mW/cm² for 30 minutes. After polymerization, the polymer coupons were removed from their cast and were heat treated at 90°C for 1 hour to evaporate unreacted monomers. The samples were sized to 5x19mm² and their edges sanded with 600 grit sand paper to even out texture introduced from sizing. Polystyrene sheets (McMaster-Carr) and nylon samples were sized and sanded in the same manner for a comparative analysis against our SMPs; Afterwards, all adsorbents were methodically washed with DI water.

SMP samples were incubated in 120ug/ml of BSA for 3 hours. Acetate buffer (pH 4.45, Ionic strength 0.01M and reagent grade materials used with filtered DI H2O) was used as the media with 0.0048% SDS and 0.0002% NAN3. The samples had a surface to volume ratio of 262mm²/ml. The BCA assay was run on all samples after the free supernatant was collected and the solution depletion method was used for quantification of protein bound.
5. Results and Discussion

5.1 CEA Characterization

5.1.1 Contact Angle and Surface Energy

Water contact angle measurements in air were performed to test the incorporation of CEA, a hydrophilic monomer, onto the polymeric surface of the SMP. Results show decreasing hydrophobicity with increasing CEA wt%. In contrast, Diiodomethane, a nonpolar reagent, was used to measure hydrophobicity at the SMP surfaces. Figure 8 shows increasing hydrophobicity with increasing CEA percentages, an opposite trend of what was shown with water. Because surfaces are highly reactive, they tend to be labile, which may begin to explain the adverse trend. The surfaces may have adapted to their environment to stay at their most thermodynamically stable state.

Surface free energy was calculated from Fowkes equation found in the methodology section. By partitioning the components of interaction forces between the solid and measuring liquid, both dispersive and polar interactions were derived although they are not completely divorced entities\textsuperscript{20}. The surface free energy results disclose the increase in both dispersive and polar forces with increasing CEA wt%. These increases are advantageous to protein adsorption as electrostatic and hydrophobic domains are the dominating interactions.
Figure 8 Contact Angle and Surface Energy Measurements of CEA SMPs

Water contact angles show decreasing hydrophobicity at the polymeric surfaces with increasing incorporation of the hydrophilic monomer, CEA.

Table 2 Contact Angle and Surface Energy Measurements: Average of Ten Trials

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>$\theta_p$</th>
<th>$\theta_d$</th>
<th>$\gamma_s^d$ (mJ/m$^2$)</th>
<th>$\gamma_s^p$ (mJ/m$^2$)</th>
<th>$\gamma_s$ (mJ/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% CEA</td>
<td>89.2 ± 5.3</td>
<td>59.11 ± 4.6</td>
<td>29.1</td>
<td>2.7</td>
<td>31.8</td>
</tr>
<tr>
<td>5% CEA</td>
<td>81.57 ± 4.2</td>
<td>49.52 ± 6.9</td>
<td>34.5</td>
<td>4.0</td>
<td>38.5</td>
</tr>
<tr>
<td>10% CEA</td>
<td>74.79 ± 7.1</td>
<td>43.8 ± 6.8</td>
<td>37.6</td>
<td>5.9</td>
<td>43.5</td>
</tr>
<tr>
<td>15% CEA</td>
<td>61.84 ± 3.9</td>
<td>15.67 ± 4.0</td>
<td>48.9</td>
<td>8.6</td>
<td>57.5</td>
</tr>
</tbody>
</table>

$\theta_p$ = Water contact angle  $\theta_d$ = Diodomethane contact angle  $\gamma_s^d$ = Dispersive surface free energy  $\gamma_s^p$ = Polar surface free energy  $\gamma_s$ = Total surface free energy

5.1.2 pH Testing

CEA was incorporated into the SMP to possess a negative charge at the polymer’s surface under physiological pH. CEA SMPs were also studied at pH 4.5 to investigate hydrogen bond influences to protein adsorption because hydrogen bonds are another major interaction force driving adsorption. To ensure hydrogen atoms were not disassociating from carboxylic acid functional groups, and were available on the surface of the CEA SMPs, the pH was monitored over the course of 4 hours. The decline of the
solution’s pH during the time course, from its baseline pH would illustrate hydrogen disassociation. Results show very little variance from the initial pH for all CEA SMP polymers. Acetate buffer was used as a control to gauge general fluctuations that may occur without the polymer influence. The acetate buffer incubated with the polymer maintains a stable pH relative to that of the acetate buffer alone. These results validate that the hydrogen atom does not disassociate from the carboxylic acid group, rendering it free for hydrogen bonding during adsorption testing.
Figure 9 pH Testing of CEA SMPs

The pH tests were carried out to measure the disassociation of hydrogen atoms from carboxylic acid functional groups on the surface of CEA SMPs. Results show the preservation of the hydrogen atom onto the polymer, thus ability to form hydrogen bonds.
5.1.3 Dynamic Mechanical Analysis

CEA SMPs were tested with dynamic mechanical analysis (“DMA”) to show preservation of the shape memory effect after CEA incorporation. All the polymers still retain their shape memory effect evidenced by the enormous temperature dependence illustrated by the 2-3 orders of magnitude drop in storage modulus at its glass transition temperature. This indicates the recovery ability of the SMP. The results display a 2-3 degree increase in the glass transition temperature per 5% CEA incorporation. The slope of the storage modulus and width of the tan delta curve represents similar shape memory recovery to the base formulation “0% CEA” or 80:20 tBA: PEGDMA. These small deviances from the base polymer are insignificant and could still provide proper mechanical properties for packing the SMP into a small capsule and recover large deformations.

![DMA Results on CEA SMPs](image)

Figure 10 DMA Results on CEA SMPs

Shape memory effect is preserved by CEA incorporation.

5.1.4 Fourier Transform Infrared Spectroscopy

FTIR spectra of the CEA SMPs show the disappearance of the alkene peak at 1680-1610cm\(^{-1}\). This validates consumption of reactive alkene groups during free radical polymerization and concurrently, convergence to alkanes through the 2850-2970cm\(^{-1}\) peak.
The reactive alkene groups were consumed during polymerization of CEA SMPs

5.2 SEM SMP Characterization

5.2.1 Contact Angle and Surface Energy

Water contact angle measurements in air display slight increases in hydrophobicity with 1% and 2.5% SEM incorporation. Due to the low density of polymers, the flip-flop of molecules on the surface is not uncommon. Atmospheric exposure and consequently adaptation, can lead to reversible changes on the surface to reduce high energy states. As such, the O-H of SEM could be embedded in the bulk of the polymer when exposed to air. 0.5% SEM has a similar hydrophobicity to 0% SEM due to the low number density of functional groups introduced. Both water and diiodomethane were used as measuring fluids to departmentalize the interfacial interaction forces into dispersion or polar components. From the contact angles alone, diiodomethane reveals the materials higher interactions with dispersive forces translating to their hydrophobic nature. Nylon has substantial polar and dispersive forces, which may be an artifact from poor manufacturing and thus surface impurities and imperfections that easily sway the results. The hydrophobicity of a material extensively influences protein adsorption as it allows water to organize loosely on the surface, granting a more energetically favorable displacement of water upon protein adsorption. A hydrophilic surface binds water tightly and would require more energy to displace water for protein. From this, the SEM polymers would encourage protein adsorption but because there are many other influential factors, contact angle measurements alone do not directly correlate to binding efficacy.
Contact angle measurements of SEM SMPs do not display SEM functional groups at the surface. Small percentages of SEM were incorporated and may fall outside of the sensitivity of contact angle measurements. This method may not display detailed chemical constituents.

Figure 12  Contact Angle and Surface Energy with SEM SMPs
Table 3  Contact Angle and Surface Energy Measurements: Average of Ten Trials

<table>
<thead>
<tr>
<th>Sample Code. Batch 1</th>
<th>$\theta_p$</th>
<th>$\theta_d$</th>
<th>$\gamma_d^p$ (mJ/m$^2$)</th>
<th>$\gamma_s^p$ (mJ/m$^2$)</th>
<th>$\gamma_s$ (mJ/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>91.2 ± 3.0</td>
<td>42.4 ± 3.3</td>
<td>18.8</td>
<td>0.4</td>
<td>19.2</td>
</tr>
<tr>
<td>Nylon</td>
<td>57.3 ± 4.4</td>
<td>46.2 ± 2.7</td>
<td>19.6</td>
<td>11.8</td>
<td>31.4</td>
</tr>
<tr>
<td>0% SEM</td>
<td>74.5 ± 3.5</td>
<td>53.4 ± 7.0</td>
<td>17.2</td>
<td>5.4</td>
<td>22.6</td>
</tr>
<tr>
<td>0.5% SEM</td>
<td>73.6 ± 4.6</td>
<td>58.3 ± 4.2</td>
<td>16.2</td>
<td>6.3</td>
<td>22.5</td>
</tr>
<tr>
<td>1% SEM</td>
<td>77.8 ± 2.5</td>
<td>55 ± 3.6</td>
<td>16.9</td>
<td>4.3</td>
<td>21.2</td>
</tr>
<tr>
<td>2.5% SEM</td>
<td>85.5 ± 2.8</td>
<td>52.3 ± 7.3</td>
<td>17.4</td>
<td>1.8</td>
<td>19.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Code. Batch 2</th>
<th>$\theta_p$</th>
<th>$\theta_d$</th>
<th>$\gamma_d^p$ (mJ/m$^2$)</th>
<th>$\gamma_s^p$ (mJ/m$^2$)</th>
<th>$\gamma_s$ (mJ/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>91.2 ± 3.0</td>
<td>42.4 ± 3.3</td>
<td>18.8</td>
<td>0.4</td>
<td>19.2</td>
</tr>
<tr>
<td>Nylon</td>
<td>57.3 ± 4.4</td>
<td>46.2 ± 2.7</td>
<td>19.6</td>
<td>11.8</td>
<td>31.4</td>
</tr>
<tr>
<td>0% SEM</td>
<td>82.4 ± 2.1</td>
<td>61.7 ± 5.1</td>
<td>15.6</td>
<td>3.4</td>
<td>18.9</td>
</tr>
<tr>
<td>0.5% SEM</td>
<td>79.2 ± 3.3</td>
<td>60.2 ± 5.3</td>
<td>15.8</td>
<td>4.3</td>
<td>20.2</td>
</tr>
<tr>
<td>1% SEM</td>
<td>83.1 ± 1.9</td>
<td>58.8 ± 2.6</td>
<td>16.1</td>
<td>2.9</td>
<td>19.0</td>
</tr>
<tr>
<td>2.5% SEM</td>
<td>83.9 ± 3.8</td>
<td>60.8 ± 2.6</td>
<td>15.7</td>
<td>2.8</td>
<td>18.6</td>
</tr>
</tbody>
</table>

$\theta_p =$ Water contact angle  $\theta_d =$ Diiodomethane contact angle  $\gamma_d^p =$ Dispersive surface free energy  $\gamma_s^p =$ Polar surface free energy  $\gamma_s =$ Total surface free energy

Surface layers have more surface free energy than their bulk because their valence electrons are not shared by their neighboring atoms$^1$. As a result, surfaces are reactive and exist in an energetically unfavorable state. In this way, surface free energy can represent the affinity of the surfaces for adsorption. Fowkes partitioned the surface free energy into two components, dispersive and polar forces. From table 3, batch 1 shows increasing dispersive or nonpolar surface free energy in the polymers with SEM incorporation. However, 2.5% SEM in Batch 2 shows a decrease in dispersive surface free energy relative to the other SEM polymers. Of the total surface free energies in the SEM polymers, the trend from greatest to lowest surface energy is 0.5% SEM$< 1%$ SEM$< 2.5%$ SEM. Since the dispersive forces are pro adsorption, these may be the ones to consider for protein adsorption. Strangely, nylon has more dispersive and polar forces than all other materials. Because non-uniform surface heterogeneity and surface mobility can alter contact angle measurements representing the material, surface energies of nylon and between SEM batches are not representative of detailed chemical compositions$^1$. 

5.2.2 Reproducibility

Between batches, the SMPs vary from approximately 0.5-8.5 degrees and can subsequently alter the trends seen within the SEM samples. The variability can be attributed to topographical imperfections on the surface, non-uniform chemical heterogeneity and/or surface mobility. Performing more measurements on a greater surface area may contribute to lowering these differences.

5.2.3 Water Equivalency Test

As evidenced with water equivalency tests, shown in figure 13, SEM was incorporated into the polymer, as the hydrophilicity increased with increasing wt % of SEM. Nylon did not equilibrate at 24 hours suggesting it could be more hydrophilic than the SEM samples. Due to its high physical and/or chemical crosslink density, diffusion of water into the polymer system is much slower. Since we performed a 3 hour time point for the binding studies, the trends before the 24 hour time point are more relevant.

![Water Equivalency Test. Batch 1](image1)

![Water Equivalency Test. Batch 2](image2)

Figure 13 Water Equivalency Test with SEM SMPs

Bulk absorption validates that the SEM monomer was incorporated into the SMP.
Table 4  Water Equivalency Test: Average of 2 Samples

<table>
<thead>
<tr>
<th>Sample Code. Batch 1</th>
<th>% Water Uptake (12 HR)</th>
<th>% Water Uptake (24 HR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Nylon</td>
<td>3.3 ± 0.0</td>
<td>5.1 ± 0.0</td>
</tr>
<tr>
<td>0% SMP</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
<td>0.5% SMP</td>
<td>4.8 ± 0.0</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td>1% SMP</td>
<td>7.8 ± 0.2</td>
<td>8.2 ± 0.3</td>
</tr>
<tr>
<td>2.5% SMP</td>
<td>10.6 ± 0.5</td>
<td>10.1 ± 0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Code. Batch 2</th>
<th>% Water Uptake (12 HR)</th>
<th>% Water Uptake (24 HR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Nylon</td>
<td>3.3 ± 0.0</td>
<td>5.1 ± 0.0</td>
</tr>
<tr>
<td>0% SMP</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>0.5% SMP</td>
<td>4.2 ± 0.2</td>
<td>4.7 ± 0.0</td>
</tr>
<tr>
<td>1% SMP</td>
<td>7.4 ± 0.0</td>
<td>7.8 ± 0.0</td>
</tr>
<tr>
<td>2.5% SMP</td>
<td>11.1 ± 0.2</td>
<td>11.0 ± 0.1</td>
</tr>
</tbody>
</table>

5.2.4 Reproducibility of Water Equivalency Test

Both batches follow the same trends and have percent differences less than 1. 2.5% SEM consistently shows more variance between batch 1 and 2 in the water equivalency and contact angle tests. The monomer mixture may have small differences of SEM content with an error in the hundredths or thousandths place.

5.2.5 Dynamic Mechanical Analysis

5.2.5.1 Shape Memory Effect

Figure 14 shows the temperature dependence of the polymers by a 1-2 orders of magnitude decrease in storage modulus at their glass transition temperatures. This transition or switching effect, allows the recovery of the polymer once it has been stored in its temporary state. The elasticity or storage modulus gives the polymer its memory and allows it to return to its original shape. Above the Tg, about 60-100°C⁰, the SEM polymers should experience immediate elasticity as all the polymers are still in their rubbery phase. The plateau of the storage modulus shown above the Tg can indicate physical cross linking or increased crystalline formations from secondary intermolecular interactions between chains¹,²,³. Since SEM introduces polarity to the SMP, more secondary intermolecular interactions between chains can take place in addition to chain realignment for crystalline/physical cross linkage. Incorporation of SEM shows
kinetically different transitions or switching rates as their slopes are moderate compared to 0% SEM. Thus, the ability of the former to recover to its memorized shape is slower. Additionally, the weak mechanical properties of the SEM polymers may hinder specific deformations or programming to its temporary shape such that manufacturing processes would need to be carefully planned out.

5.2.5.2 Storage Modulus

SEM incorporation leads to a reduced storage modulus below Tg. Because chemical constituents have the largest effect on altering thermal mechanical properties, it is reason to believe the C-O bonds from SEM allow for increased chain/bond flexibility below the Tg\(^1\). Tert-Butyl Acrylate (tBA) is also displaced by the weight percent of SEM during monomer mixture preparations, contributing to lower Tg/storage modulus values. Less free volume from the bulky tert-Butyl groups of tBA restrict large molecular movements and as such, more energy requirements are needed for thermo mechanical transitions.

0.5 and 2.5% SEM have similar glass transition temperature slopes, regressing more gradually from their glassy to rubbery phase relative to 0% SEM while 1% SEM has the slowest transition phase. The storage modulus of 1% SEM is the highest of the SEM incorporated polymers, theoretically possessing better elastic properties for shape recovery. 0.5 and 2.5% exhibit similar storage moduli and switching stages but in batch two, 2.5% SEM possesses better elastic recovery than 0.5%. This may be a reproducibility error and should be repeated. The trends within the SEM samples are less evident, but may be contributed to the polymerization process itself as molecular weight, crystallinity and cross link density affect the storage modulus and Tg values as well as chemical composition\(^1,3\).

5.2.5.3 Tan Delta Peak

SEM incorporation into the SMP causes decreased crosslink density, increased crystallinity as seen by the lower amplitude, and a left shift of the tan delta peak relative to the 0% SEM polymer. Thus, SEM polymers have less mechanical strength or damping abilities that may permit low impact breaks within the polymer system. Additionally, the broad peaks of the SEM polymers dictate slower glass transition phases, in tune with the storage moduli slopes. Two transitions are seen in 0.5% SEM that may be a caustic response from the annealing of polymeric chains through increased molecular movement after its first glass transition phase. The additional secondary intermolecular interactions yield crystallinity above
the first glass transition temperature but can slip as the temperature increases further illustrating the second glass transition temperature\textsuperscript{1}. 2.5\% SEM has the greatest cross linking density and amorphous content compared to the other SEM incorporated polymers and thus has better damping abilities at its glass transition temperature. The tan delta peak of 1\% SEM may indicate a more uniform increase in molecular weight during its polymerization process as it transitions slowly from its glassy to rubbery state with a tan delta peak spanning the largest temperature range.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{DMA_results.png}
\caption{DMA Results on SEM SMPs}
\end{figure}

DMA storage modulus and tan delta graphs of SMPs with 0, 0.5, 1 and 2.5 wt\% incorporation of SEM. The SEM samples have depreciated mechanical and shape memory properties.
Table 5  Thermal Mechanical Properties of SEM SMPS: Average of Two Trials

<table>
<thead>
<tr>
<th>Sample Code. Batch 1</th>
<th>Glassy Modulus (Mpa)</th>
<th>Rubbery Modulus (Mpa)</th>
<th>Tan Delta Peak</th>
<th>Tg (C°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% SEM</td>
<td>1551.45 ± 473.41</td>
<td>4.12 ± 0.08</td>
<td>1.75 ± 0.00</td>
<td>51.87 ± 1.21</td>
</tr>
<tr>
<td>0.5% SEM</td>
<td>850.56 ± 31.28</td>
<td>5.03 ± 0.17</td>
<td>0.52 ± 0.00</td>
<td>29.63 ± 1.02</td>
</tr>
<tr>
<td>1% SEM</td>
<td>1214.05 ± 264.81</td>
<td>5.27 ± 0.59</td>
<td>0.52 ± 0.02</td>
<td>40.6 ± 0.59</td>
</tr>
<tr>
<td>2.5% SEM</td>
<td>650.33 ± 9.61</td>
<td>4.47 ± 0.08</td>
<td>0.78 ± 0.16</td>
<td>32.39 ± 5.42</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Code. Batch 2</th>
<th>Glassy Modulus (Mpa)</th>
<th>Rubbery Modulus (Mpa)</th>
<th>Tan Delta Peak</th>
<th>Tg (C°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% SEM</td>
<td>1817.20 ± 195.73</td>
<td>4.35 ± 0.07</td>
<td>1.72 ± 0.03</td>
<td>52.33 ± 2.35</td>
</tr>
<tr>
<td>0.5% SEM</td>
<td>718.41 ± 41.21</td>
<td>4.81 ± 0.08</td>
<td>0.52 ± 0.00</td>
<td>30.28 ± 0.26</td>
</tr>
<tr>
<td>1% SEM</td>
<td>1212.45 ± 71.63</td>
<td>5.32 ± 0.10</td>
<td>0.48 ± 0.01</td>
<td>42.29 ± 0.42</td>
</tr>
<tr>
<td>2.5% SEM</td>
<td>1015.69 ± 254.29</td>
<td>5.15 ± 0.80</td>
<td>0.74 ± 0.01</td>
<td>34.01 ± 1.11</td>
</tr>
</tbody>
</table>

Figure 15  Thermomechanical Reproducibility Analysis

The stiffness between batches is the most variable but the other properties (Tg and tan delta peak) have good reproducibility.
5.2.5.4 Reproducibility of Thermomechanical Properties

Figure 15 illustrates reproducibility of thermo mechanical properties between batches. The glassy modulus is most prone to fluctuations between batches. Literature suggests these variations may be due to the delicate process of polymerization, including pre and post processing. Variables such as aging, atmospheric exposure and post heat treatments after polymerization can contribute to molecular rearrangements and alter mechanical properties.\textsuperscript{1, 3} The tan delta peak and Tg between both batches have similar trends with relatively low standard deviations. However, the rubbery moduli of 2.5% SEM polymers potentially elicit different trends of stiffness in account of the standard deviations. Since binding occurred at this rubbery state and stiffness is an influential factor in protein adsorption, this small trend change could directly affect binding abilities and could start to explain the variable binding trend between batches for 2.5% SEM. If heat pockets were introduced in the polymer coupon during the polymerization process by the UV source, non-uniform cross linkage, molecular rearrangements and stresses could have occurred in the 2.5% samples leading to different thermo mechanical properties. Note that the coupons used for DMA analysis were the same coupons used to prepare the samples for the binding experiment.

An important distinction in the batches is the modulus switch between the 2.5% SEM and 0.5% SEM sample in batch 2. In batch one, the 2.5% and 0.5% SEM samples are similar but the glassy and rubbery modulus of 2.5% is slightly lower than 0.5% SEM. However, in batch 2, the glassy moduli and glass transition temperature phase of 2.5% SEM is higher than 0.5%. Since stiffness could play a role in adsorption, this may be a reason in a drop in adsorption at 2.5% SEM in batch 1.

5.2.6 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy was utilized to confirm complete polymerization. The SEM polymers show the disappearance of a peak near 1610-1680cm\textsuperscript{-1}, the alkene range. Additionally a large peak is shown in the 2850-2970cm\textsuperscript{-1} range, depicting convergence of alkene bonds to alkane bonds.
Figure 16 FTIR on SEM SMPs

The reactive alkene groups during SEM polymerization were consumed.
5.3 Adsorption Testing

5.3.1 Experiment 1. MA

Figure 17 shows the adsorption ability of MA at 5 and 15 wt% compared to the non-functionalized base polymer (0%) to poly-L-amino acids. poly-L-arginine was used as an EDGP analog with a PI of 10.76 and poly-L-glutamic acid was introduced as a negative control with a PI of approximately 3. Significant binding onto the polymeric surfaces did not occur. Although the 15% MA SMP condition shows adsorption of poly-L-glutamic acid during the 2 hour time point, its binding is variable. One explanation of why poly-L-glutamic acid may bind to the modified polymer is the presence of counter ions from PBS binding to the surface of the polymer first, encouraging poly-L-glutamic acid to bind secondary onto the new monolayer. The large error bars and negative values indicate the levels of binding are beneath the sensitivity threshold of the assay. 5 minute and 30 minute incubations were inaccurate time points as the post and pre incubations of SMP in protein solution were of the duration of the time point itself or exceeded beyond this point. Since MA is a small monomer with its functional site near its vinyl group steric hindrance may attenuate binding. In essence, because of the pure randomness and noise from the assay, a better monomer may contribute to higher levels of detectable binding coupled with an increase in surface area to provide more available binding sites for binding.
Figure 17  Experiment 1 MA

Time points of poly-L amino Acids adsorbed onto MA SMPs (Surface to volume ratio: 37.68mm$^2$/ml, poly-L-arginine concentration: 44ug/ml, pH 7 and temperature: 37°C).

5.3.2 Experiment 2-3. CEA

Figure 18 shows Poly-L-amino acid adsorption onto CEA SMP surfaces or nylon. Since the base material of the esophageal string test is made from nylon, it was introduced in the binding experiment for a comparative analysis against the modified SMP samples. Experimental repeats were increased from 3 to 6 and the surface-area-to-volume ratio was increased approximately 3 fold to decrease the error and to supply more binding support for detectable binding. As such, the standard deviations and negative values still exist. Two alternative approaches to have detectable binding are by: 1) picking a more sensitive detection method or 2) increasing the surface area further. The data shows no appreciable amounts of binding with CEA incorporation. Although the base formulation of the SMP (0%) mildly binds poly-L-arginine, the results of this are variable. Nylon also does not show detectable amounts of binding suggesting the esophageal string test may work in capturing proteins through high specific surface area.
Therefore, increasing the surface area further for all samples was employed in the next experiments to ensure enough support for detectable binding.

**Figure 18** Experiment 2-3. CEA

Time points of poly-L amino acids adsorbed onto CEA SMPs and/or nylon (surface- to- volume ratio: 94.2mm²/ml, poly-L-arginine concentration: 44ug/ml, pH 7 and temperature: 37°C⁰).

5.3.3 Experiment 4. Surface Area 132mm²/ml

High specific surface area is important as it relates to the total loading capacity of the polymer for protein. As such, figure 19 summarizes the trials of surface areas examined (some of which are outlined below). SMP particles were also created but exhibited no observable appreciation in poly-L-amino acid adsorption.
Figure 19  Surface Areas Implemented to Maximize Loading Capacity

Figure 20 displays poly-L-arginine binding onto SMP (80:20- tBA: PEGDMA) and nylon samples. The surface area was increased to 2640mm$^2$ per condition from 188.4mm$^2$ per condition to absorb more protein onto the surface. The surface to volume ratio only increased from 94.2mm$^2$/ml to 132mm$^2$/ml because more volume was needed to cover the larger samples. Nonetheless, after increasing the surface area beyond previous experiments, there are still negative readings and large standard deviations. The assay sensitivity threshold could be an avenue to consider but because the surface to volume ratio was not much more than the previous experiment, additional efforts were made to minimize the volume the adsorbents were incubated in and increase the surface area for the next experiments.

![Figure 20](image)

**Figure 20** Experiment 4. Surface Area 132mm$^2$/ml

Poly-L amino acids adsorbed onto SMP and nylon (surface- to- volume ratio: 132mm$^2$/ml, poly-L-arginine concentration: 44ug/ml, pH 7 and temperature: 37C$^0$).
5.3.4 Experiment 5. Surface Area with SMP Particles

Polymer particles were created to increase the loading capacity for the protein far beyond the surface areas introduced in the previous experiments. High surface areas are usually necessary for implementing adsorption. Many studies use particulate or beaded adsorbents for large surface area exposure, for instance, Sepharose 4B (agarose beads) used for adsorption in chromatographic methods have a surface area of $8\text{m}^2/\text{ml}$\textsuperscript{13}. The largest surface to volume ratio achieved thus far is $0.132\text{m}^2/\text{ml}$, reaching nowhere near this level. To attain a quantifiable amount of protein binding onto our polymeric surface, particulates may need to be created. Even nylon has not adsorbed appreciable amounts of protein, and since this is the material the esophageal string test is made from, known to capture protein, the small surface area may be the underlying issue. Therefore creating particles was the new concentration. A dremel was used to create the adsorbent particles but in the process, various particle sizes were generated. Figure 21 and 22 display the large range of the sizes created (.01-1.2mm SMP and 0.1-13.857mm nylon). Because nylon sample sizes were significantly larger than the SMPs, they could not be used for comparison during the binding experiments. These SMP particles were washed several times and centrifuged to remove extremely small particles that could be difficult to remove after the binding procedure. Centrifugation should pool the larger particles to the bottom, and the residing supernatant should contain the smaller sized particles. This supernatant was decanted to remove these small particles. After the particles were correctly processed and dried, the adsorption experiment was carried out and the results are displayed in figure 23.
**Figure 21 SMP Particles**

80:20 (tBA: PEGDMA) SMP particles imaged through an optical microscope. Large ranges exist; some particles are below the detection limit of the scope. The sizes ranged from 0.01-1.2mm. Particles smaller than 0.01mm could not be measured using this method.
Nylon particles imaged through an optical microscope. Nylon particles are larger than SMP; therefore, the two cannot be compared in further polymer particle adsorption studies. The sizes ranged from 0.1-13.857mm. The exact surface area per condition is unknown due to the large variation of sizes created during the generation of the SMP samples. As such, the weight of the particles was used to control for a fixed amount per condition. The most saturated samples with the polymer particles were at 0.06g and a condition at 0.04g was also tested. Poly-L-amino acids were the study protein at a pH of 7 in PBS buffer. The results show that even though the surface to volume ratio is maximal in this experiment relative to the others, appreciable amounts of binding did not occur. The problems associated with creating SMP particles is the high variability of particulate size, the uncertainty of false negative results, the aggregation of the hydrophobic particles together in solution, and the high buoyancy of the particles. It can be speculated that the large error bars resulted from the large range of sizes produced when creating the SMP particles. Initially, the particles were passed through a 230um mesh sieve to try to eliminate some of this variation but most of the sample sizes were so small that >80% of the particulate passed through. Thus, centrifugation was implemented to remove the particles but further optimization to create tighter particle sizes should be implemented. There is uncertainty that all the small particles were in fact removed by this method. This can lead to false negative results if the particles have bound protein on its surface and the
solution depletion method is performed. In this method, we only want to measure free bulk protein that was not bound to the polymer surface and total protein. The difference of the two values yield the protein bound to the polymer surface. If the polymer particle with protein bound is not properly removed from the free protein being measured, there is a large possibility of a false negative result. The shape of the polymers also added variability to each condition. Another concern is full exposure of the particles to the protein solution. The purpose of creating SMP particles was to increase surface area, but because of the hydrophobicity of the polymers, they clumped together to reach a more thermodynamically favorable state during aqueous incubation. The SMP particles also have a density similar to water such that they tend to float in PBS. Together, these findings show that the total surface areas of all the particles are not available for the protein to adsorb, defeating the overall purpose in creating these particles. One solution to this problem is forcing the solution to pass through all the particles. This can be done by a rotary device or through vigorous shaking in a closed vial bringing us to experiment 6 where this was carried out.

<table>
<thead>
<tr>
<th>Amino Acid adsorbed. 1 HR Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-L-Arginine</td>
</tr>
<tr>
<td>Poly-L-Glutamic Acid</td>
</tr>
</tbody>
</table>

![Figure 23](image)

**Figure 23** Experiment 5 Surface Area with SMP Particles

Poly-L amino acids adsorbed onto 0.04g and 0.06g of SMP (poly-L-arginine concentration: 44ug/ml, pH 7 and temperature: 37°C).

**5.3.5 Experiment 6. Surface Area with SMP Particles- Complete Submersion**

Figure 24 displays poly-L-amino acid adsorption onto SMP particles with an increase in surface-to-volume ratio. The particles were incubated in a closed vial and were vigorously shaken throughout the incubation period to ensure full exposure of the polymeric particles to the protein solution. However, minimal adsorption took place with a high deviation for poly-L-arginine. Because poly-L-glutamic acid has a negative value but a low standard deviation, this may be the limit of detection of the assay. It is still not
clear if the particles were removed before the BCA assay was performed which could interfere with the results as a false negative reading. Therefore, we reverted back to creating square samples to increase the surface area.

Figure 24 Experiment 6 Surface Area with SMP Particles-Complete Submersion

Poly-L amino acids adsorbed onto 0.122g of SMP (poly-L-arginine concentration: 44ug/ml, pH 7 and temperature: 37°C).

5.3.6 Experiment 7. Surface Area 648mm²/ml

Figure 25 displays protein adsorption after exposing 648mm² SMP surface area per 1mL of protein solution. Poly-L-glutamic acid, the negative control binds to the polymeric surface better than poly-L-arginine. Our aim is to specifically bind highly cationic proteins of EoE onto the SMP through anionic charged groups on the polymer surface. Because the polymer used for this experiment was the base formulation with mostly hydrophobic bonds, it will bind protein more nonspecifically. In addition, glutamic acid was attached to sodium as purchased from Sigma. The sodium’s positive charge allows this to bind to the negative groups on the polymer surface negating its repulsion and serving as a negative control. After performing several trials to increase the protein capacity of the polymer with little improvement, surface area considerations, although important, may not be the factor causing minimal binding.
Poly-L-Amino Acid adsorbed. 1 HR Incubation

![Graph showing protein adsorption on SMP and Nylon](image)

**Figure 25** Experiment 7 Surface Area 648mm²/ml

Poly-L amino acids adsorbed onto SMP and nylon (Surface area to volume ratio: 648mm²/ml, poly-L-arginine concentration: 44μg/ml, pH 7 and temperature: 37°C).

After more consideration for maximizing surface to volume ratios, the best approach was submerging 5x12x1mm SMP sample in 1mL volume, specifically in a 1.5mL eppendorf tube. This combination allows the entire polymer surface to be submerged in solution and does not consume expensive processing time with creating numerous coupons. The problem associated with incubating numerous SMP samples in one condition is the uncertainty of a fully exposed surface. The polymers lie on top of each other and can hide a large surface area. One solid SMP support eliminates this issue and still exposes large surface area to the protein solution.

Many studies refer to hydrophobic forces as the dominate interactions for binding proteins more firmly. Dehydration of the polymer and protein surfaces is a prerequisite for protein adsorption. Water that resides on the surface of both the hydrophobic protein domains and hydrophobic polymer surfaces are highly ordered (loosely bound) and require less energy to displace than with hydrophilic surfaces where the water is tightly bound. In this way, hydrophobic domains increase polymer-protein interactions and trend toward irreversible binding. The hydrophobic forces were considered for the polymer surface but the protein needs complimentary hydrophobic domains for the interaction to take place. Poly-L-arginine was chosen because of its net positive charge that resembles the unique cationic biomarkers of EoE. However, the synthetic analog does not emulate physiological proteins; it is just composed of one type of amino acid. Although the protein’s charged outer surface allows specific binding onto the anionically charged polymer surface, it lacks a hydrophobic core to create long lasting, densely packed contacts. Therefore, the study protein was changed to native EDGPs for the next series of experiments. The
diversity of amino acids, particularly hydrophobic domains, and their increased interaction forces can facilitate irreversibly binding.

5.3.7 Experiment 8. Eosinophil Lysate

Eosinophil cationic protein, eosinophil derived neurotoxin and major basic protein 1 were tested for adsorption onto SMP’s without functional group incorporation. We decided to optimize protein adsorption by using the base SMP formulation because of its ability to preference cationic proteins because of its slight negative charge from the crosslinker, PEGDMA. The base SMP formulation also facilitates hydrophobic interactions from tBA methyl groups (CH3). For interpretation of protein binding to our polymer surface, amino acid analysis was performed for each protein. Protein properties listed below in table 6 can affect surface interactions during adsorption. Since there are many protein adsorption factors it is hard to predict which ones are most influential in protein adsorption, thus 3 of the 4 proteins were quantified to determine binding. For instance, Larger molecules tend to bind better because they have more sites of contact for the polymer surface. ECP, EDN and MBP1 have small molecular weights, reducing the strength of the interaction to the polymeric surface. ECP, EPX and MBP1 all share high cationic charges that creates a looser structural construct from the repulsion between adjacent charged residues. Thus, the internal mobility needed upon adsorption and relaxation is greater for these proteins, encouraging protein adsorption. EPX and MBP1 have the highest quantity of disulfide bonds that ultimately hold the protein in its tertiary structure and aid in its stability, a discouraging protein adsorption factor.
Table 6  Protein Characterization

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>% Hydrophobicity</th>
<th>% Charged Sites</th>
<th># disulfide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECP</td>
<td>18.39</td>
<td>11.4</td>
<td>43.16</td>
<td>20.63</td>
<td>9</td>
</tr>
<tr>
<td>EDN</td>
<td>18.35</td>
<td>8.9</td>
<td>41.62</td>
<td>15.53</td>
<td>9</td>
</tr>
<tr>
<td>EPX</td>
<td>81.04</td>
<td>10.8</td>
<td>43.24</td>
<td>24.2</td>
<td>17</td>
</tr>
<tr>
<td>MBP1</td>
<td>25.21</td>
<td>10.9</td>
<td>40.54</td>
<td>26.12</td>
<td>12</td>
</tr>
</tbody>
</table>

After performing adsorption testing, select EDGP binding onto the surface of the methanol treated SMP (80:20 tBA: PEGDMA) was quantified. ECP binds both nylon and SMP polymers the best over EDN and MBP1. Two reasons why ECP may bind preferentially is its high isoelectric point and low disulfide bond count relative to the other proteins. These factors place it in a looser construct with increased internal mobility. To fully study this binding phenomenon, one variable should be eliminated that could cause increased binding through pore size and not through chemical constituents on the surface. The polymer was methanol treated for 48 hours, which causes substantial swelling of the polymer. This affect could stretch pore sizes beyond recovery through the dehydration step. To eliminate pore size from interfering with binding, SMP samples were heat-treated in lieu of methanol treatments to rid of unpolymerized monomers.

Figure 26  Experiment 8 Eosinophil Lysate. ECP Adsorption

Eosinophil cationic protein (ECP) adsorption onto SMP methanol treated samples (Surface area to volume ratio: 154mm$^2$/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C$^0$, Donor 1).
Table 7  % Protein Bound Relative to Total ECP

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>63.51 ± 20.35</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>50.23 ± 11.85</td>
</tr>
</tbody>
</table>

Figure 27  Experiment 8 Eosinophil Lysate. EDN Adsorption

Eosinophil derived neurotoxin (EDN) adsorption onto SMP methanol treated samples (Surface area to volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C⁰, Donor 1).

Table 8  % Protein Bound Relative to Total EDN

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>2.89 ± 0.04</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>2.89 ± 0.02</td>
</tr>
</tbody>
</table>

Figure 28  Experiment 8 Eosinophil Lysate. MBP1 Adsorption

Major Basic Protein 1 (MBP1) adsorption onto SMP methanol treated samples (Surface area to volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C⁰, Donor 1).

Table 9  % Protein Bound Relative to Total MBP1

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>1.91 ± 0.07</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>1.96 ± 0.12</td>
</tr>
</tbody>
</table>
5.3.8 Experiment 9-10. Eosinophil Lysate

After changing the post processing method of the SMP from methanol treatments to purely heat treatments, appreciable amounts of binding did not occur. This suggests that if methanol did alter the polymer’s pore size, ECP may have been absorbed through the pores or adsorbed at the surface of increased surface area. The adsorption mechanism is unknown but the results below imply it was not a function of purely non-covalent binding of the equivalent surface area. The % protein bound was normalized to each donor’s specific total protein content such that comparisons between donors could be made. That said, after comparing nylon between experiments, it has variable binding which could be because of the adsorbent itself or reproducibility error of the assay. Nylon exhibited approximately 30% more ECP binding in experiment 9 than experiment 8 but dropped to nearly 58% comparing experiment 9 to 10. Nylon was purchased with surface scratches from the manufacturer, altering uniformity from sample to sample. This alone could influence adsorption as the scratches could facilitate sites for physical entrapment of the protein and increased surface area. EDN and MBP1 minimally bind to the polymer surface and do not need to be repeated again. To test the reproducibility of the ECP assay and if methanol treatments sincerely encourage binding, the experiment was performed once more with both heat-treated, methanol treated and nylon samples.
**Figure 29** Experiment 9 Eosinophil Lysate. ECP Adsorption

Eosinophil cationic protein (ECP) adsorption onto SMP heat-treated samples (Surface area to volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C, Donor 1).

**Table 10** % Protein Bound Relative to Total ECP

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>8.61 ± 1.92</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>86.21 ± 8.49</td>
</tr>
</tbody>
</table>

**Figure 30** Experiment 9 Eosinophil Lysate. EDN Adsorption

Eosinophil derived neurotoxin (EDN) adsorption onto SMP heat-treated samples (surface area-to-volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C, Donor 1).

**Table 11** % Protein Bound Relative to Total EDN

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>0.43 ± 0.00</td>
</tr>
</tbody>
</table>
**Figure 31** Experiment 9 Eosinophil Lysate. MBP1 Adsorption

Major Basic Protein 1 (MBP1) adsorption onto SMP heat treated samples (surface area-to-volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C, Donor 1)

**Table 12** % Protein Bound Relative to Total MBP

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>0.77 ± 0.03</td>
</tr>
</tbody>
</table>

**Figure 32** Experiment 10 Eosinophil Lysate. MBP1 Adsorption

Eosinophil cationic protein (ECP) adsorption onto SMP heat-treated samples (surface area-to-volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C, Donor 2).

**Table 13** % Protein Bound Relative to Total ECP

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>3.71 ± 1.71</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>27.90 ± 7.30</td>
</tr>
</tbody>
</table>
Figure 33  Experiment 10 Eosinophil Lysate. EDN Adsorption

Eosinophil derived neurotoxin (EDN) adsorption onto SMP heat- treated samples (surface area- to- volume ratio: 154mm$^2$/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C⁰, Donor 2).

Table 14  % Protein Bound Relative to Total EDN

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>0.43 ± 0.03</td>
</tr>
</tbody>
</table>

5.3.9 Experiment 11. Eosinophil Lysate

Minimal ECP adsorption onto SMP was found for both heat-treated and methanol treated samples. ECP bound the nylon samples at a higher degree but again, this could be because of slight imperfections that exist on the nylon surface. The results for the methanol treated SMPs were not reproducible. This may be explained by delicate changes between the methanol treatments for the two experiments, as the smallest change in polymer processing could alter the surface severely.

Going back to the literature, adsorption is maximal if the pH of the solution is equal to or near the isoelectric point of the protein$^{5,7,8,12}$. This can be explained in a couple of different ways. The protein and polymeric surface should not be thought of in isolation, rather protein–protein interactions occur frequently in addition to polymer-protein interactions. In our study, with a pH of 7, the eosinophil derived granule proteins are positively charged and can attribute lateral repulsion upon binding to adjacent binding sites; the proteins may need to be uncharged to decrease protein-protein interactions and promote polymer-protein interactions. In addition, charged proteins take up more occupancy onto the polymer surface, reducing the amount of protein that can bind per meter squared of surface area.

Studies have also shown that adsorbents introduced to multi component protein solutions tend to adsorb one protein or molecular species preferentially$^{13}$. This is dependent on the concentration and
affinity of the protein for the polymeric surface. If the concentration of one protein is higher than the rest, it will have a higher probability of interacting with the surface and adsorbing over rare proteins in the mix. Increased concentration also promotes stacking of proteins next to one another by decreasing the residency time of the protein onto the polymer surface, discouraging over-relaxation states that could otherwise take up ample space. As such, the concentration of the study protein was increased to 1 mg/ml to decrease residency time on the polymer surface. For the affinity of a protein, if one protein has the correct surface amino acids that compliment the interaction forces produced by the polymer surface, that protein will bind preferentially. In the last set of experiments with the EO lysate, a pool of EO proteins were competing for the surface but the media itself was infused with 8% FBS that could have bound preferentially as well. Thus, in the next experiment, only purified protein was introduced so we could study the mechanism involved in protein adsorption and control for competition that may be taking place and altering adsorption of the EDGPs.

The study protein was also changed to BSA. The main justification is that BSA is frequently used as the study protein for adsorptions studies. BSA is well characterized, has the high internal mobility, is a moderately sized protein that could bind irreversibly and is easily available in pure delipidated form. Below are its specific characteristics that aid in its pro-adsorption characteristics. In correlation, the solution was switched from PBS to acetate buffer with a pH of 4.5 to bring the net charge of BSA to zero.
Table 15  BSA Characterization

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>% Hydrophobicity</th>
<th>% Charged Sites</th>
<th># disulfide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>69.29</td>
<td>4.7</td>
<td>42.83</td>
<td>33.27</td>
<td>35</td>
</tr>
</tbody>
</table>

Figure 34  Experiment 11 Eosinophil Lysate. ECP Adsorption

Eosinophil cationic protein (ECP) adsorption onto SMP heat-treated samples (surface area- to- volume ratio: 154mm²/ml, EO lysate conc.: 500k/ml, pH 7 and temperature: 37°C⁰, Donor 3).

Table 16  % Protein Bound Relative to Total ECP

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP + Lysate</td>
<td>0.56 ± 0.26</td>
</tr>
<tr>
<td>SMP Methanol + Lysate</td>
<td>2.04 ± 1.41</td>
</tr>
<tr>
<td>Nylon + Lysate</td>
<td>27.00 ± 2.29</td>
</tr>
</tbody>
</table>

5.3.10 Experiment 12. CEA

After incorporating COO⁻ functional groups to the polymeric surface, switching the study protein to BSA, increasing the concentration to 1mg/ml and adjusting the pH of the solution buffer to the pI of the study protein, no protein was found bound to the CEA SMPs shown in figure 35. One key consideration of why the no binding occurred onto the CEA polymers is the high ionic strength of the solution. The ionic strength of the acetate buffer used was at 0.1M. This can have an enormous degree of control over protein adsorption as the counter ions in solution weaken electrostatic interactions both on the protein and polymer surfaces by competing with the same binding sites. Since these molecules are small, they are transported to these sites on the protein and polymer faster and can consume critical sites ultimately influencing adsorption and adsorption kinetics⁷. Therefore, for future experiments, the ionic strength was adjusted to 0.01M to correct for any competing counterions.
Since the mechanism of adsorption is complex, and there are a multitude of factors that could ultimately influence this mechanism, a positive control was needed to test if the correct experimental parameters were set in place for pro-adsorption. Otherwise, the iterations of system adjustments could go beyond the period of this project.

![BSA Adsorption](image)

**Figure 35** Experiment 12 CEA

Bovine serum albumin (BSA) adsorption onto SMP samples (surface area- to-volume ratio: 154mm$^2$/ml, conc.: 1mg/ml, pH 4.5 and temperature: 37°C).

### 5.4 Positive Control – Optimization of Polystyrene Microspheres

Polystyrene microspheres are an established adsorbent utilized in numerous studies to investigate protein adsorption. As such, 0.51um microspheres were purchased to validate experimental parameters set forth previously and serve as a positive control. These spheres have a high specific surface area and are hydrophobic with a smooth surface to promote protein adsorption.

#### 5.4.1 Surface Area and Surfactant Optimization

The surfactants in which the microspheres were suspended in, were diluted several folds to find the best surface area- to- surfactant ratio for maximal adsorption. Results show 92.4% adsorption of BSA to polystyrene microspheres with a 1:100 dilution, the best choice for further adsorption studies with these spheres. Though the 1:1000 dilutions yielded more protein bound per surface area, more error was associated with this condition. One reason for this may be because of the minimal amounts of SDS in solution. SDS prevents the microspheres from clumping. The hydrophobic end of the surfactant resides on the polymers surface while the flanking end is negatively charged creating a negatively charged polymeric surface. The negative charges of the polymer particles cause repulsion from each other, thus controlling the
dispersion of the polymers in solution. The high standard deviation could be a result of non-uniform clumping from sample to sample and thus more error in pipetting the spheres to the protein solution for the actual binding experiment. Diluting the polymers ultimately leave more room on the polymer surface for protein adsorption but can also cause polymer-polymer interactions as these spheres are free floating.

Absolute BSA adsorbed of the 1:10 and 1:1000 diluted microspheres amounted to only 0% and 3.06%. The final SDS concentration of the 1:10 dilution was 0.048%, high enough to interfere with binding. Because SDS is an anionic surfactant, it could occupy the binding sites of the polymer or protein, this illustrates that too much SDS is not good either for our purposes. The 1:1000 dilutions reduce the surface area for protein to adsorb onto which may be the reason for such a low adsorption yield. Here, only $5.57 \times 10^8$ microspheres were added per condition compared to the 1:100 dilutions where $5.5760 \times 10^9$ microspheres were added. The 1:100 diluted microspheres seem to meet the perfect balance between surface area and surfactant levels.

**Figure 36** Surface Area and Surfactant Optimization

Optimal surface area-to-surfactant ratios for BSA adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:10: 185263mm$^2$/ml, surface area-to-volume ratio 1:100:1852mm$^2$/ml, surface area-to-volume ratio 1:1000:18.52mm$^2$/ml, BSA conc.: 120ug/ml, pH 4.5 and temperature: 37C$^0$).
Table 17  % Protein Bound Relative to Total BSA

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Bound- Surfactant 1:10</td>
<td>- 10.23 ± 0.25</td>
</tr>
<tr>
<td>% Bound- Surfactant 1:100</td>
<td>92.40 ± 1.47</td>
</tr>
<tr>
<td>% Bound- Surfactant 1:1000</td>
<td>3.06 ± 1.76</td>
</tr>
</tbody>
</table>

5.4.2 Total Exclusion of Surfactants

To clarify if the same optimal surface area to volume ratio (1852mm²/ml) without any interference of surfactants would bind even more, microspheres were washed once in acetate buffer to rid SDS from solution. Interestingly, adsorption decreased by approximately 85%. This suggests a role surfactants may have in adsorption if the correct balance exists. SDS, the anionic surfactant the microspheres were shipped in, may have a role in attracting the BSA near the interfacial region of the polymer since the surfactants are utilized to charge the microspheres and prevent clumping. Adversely, free SDS could weaken intramolecular forces of protein providing structural mobility. In any event, the microspheres were not washed but only diluted 1:100 in future experiments.

Figure 37  Total Exclusion of Surfactants

Washed polystyrenes affect on BSA adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:100:1852mm²/ml, BSA conc.: 120ug/ml, pH 4.5 and temperature: 37°C).

Table 18  % Protein Bound Relative to Total BSA

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed polystyrene beads</td>
<td>6.73 ± 0.98</td>
</tr>
</tbody>
</table>
5.4.3 BSA Concentration Optimization

Next, the optimal concentration of BSA was tested. One source discussed the optimal protein concentration range for adsorption studies to be 200-400ug/mL\(^{12}\). Thus, the concentrations were chosen within this range. As mentioned before, concentration can determine the residency time a protein spends on the polymer surface and as such, the degree to relaxation of the protein. A more packed orientation could aid in more binding. Results show higher binding per meter squared for high protein concentrations with ~52ug of protein bound where the low protein concentration had approximately 25ug bound. From these results, the high protein concentration was used in the next experiments.

It should be noted that the adsorption dropped in the 120ug/ml BSA samples by nearly 35% relative to the surface area to surfactant experiment with the same experimental parameters. The only minute alteration was the preparation of the acetate buffer wherein it was prepared from DI water passed through a 0.2um filter before, the acetate buffer was made from nano-pure water in this experiment. The next parameter of optimization tests these influences.

![Protein Concentration Influence on Protein Adsorption](image)

**Figure 38** BSA Concentration Optimization

Optimal bovine serum albumin (BSA) concentration for maximal adsorption onto polystyrene microspheres (surface area- to-volume ratio 1:100:1852mm\(^3\)/ml, BSA conc.: 120ug/ml, pH 4.5 and temperature: 37\(^\circ\)C).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Concentration</td>
<td>56.52 ± 5.21</td>
</tr>
<tr>
<td>Low Concentration</td>
<td>63.47 ± 1.98</td>
</tr>
</tbody>
</table>

**Table 19** % Protein Bound Relative to Total BSA
5.4.4 Buffer Solution Optimization

The buffer solution was tested to see if its water base composite effected adsorption. Acetate buffer was made from nano-pure water or filtered DI water. Since the microspheres are highly reactive and are prone to adsorb nonspecifically to ions, organic species and microbes that could be contaminants of the water, the degree to this effect was examined. The results show that the microspheres suspended in acetate buffer made from filtered water does adsorb BSA better than the acetate buffer made from nano-pure water. Therefore, acetate made from filtered water was implemented in the future studies. However, the degree to binding depreciated much further. The variability amongst the positive control illustrates how sensitive adsorption is and the degree of detail that needs to be considered. Numerous factors could have prevented maximal protein binding on either the solution, protein or polymer end. For this reason, the study was continued by examining one potential cause, pipetting error.

![H2O Influence (in Solution) on Adsorption](image)

**Figure 39 Buffer Solution Optimization**

Optimal purity for Bovine Serum Albumin (BSA) adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:100; 1852mm²/ml, BSA conc.: 120ug/ml, pH 4.5 and temperature: 37°C).  

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano-Acetate Buffer</td>
<td>8.67 ± 2.31</td>
</tr>
<tr>
<td>Filtered Acetate Buffer</td>
<td>19.71 ± 3.18</td>
</tr>
</tbody>
</table>
5.4.5 Reproducibility Testing

The microspheres have a high initial concentration of $1.394 \times 10^{12}$ microspheres per ml. The total amount of microspheres after a 1:100 dilution, introduced to each experimental condition, amounts to only 4ul of the undiluted stock. Pipetting could contribute to enormous error if the spheres were clumped together or not vortexed properly to disperse the spheres and get a uniform amount each time. The degree to pipetting error was measured to see if this was problematic once methodical vortexing was controlled for. The microspheres had an 8% difference of BSA adsorption when larger volumes were aspirated over smaller volumes. Thus, larger sample dilutions were prepared for future experiments. The exact reason why BSA adsorption decreased substantially previously is unknown, but meticulous precautions was taken to treat all the base parameters the same.

![Figure 40 Reproducibility Testing](image)

Reproducibility of Bovine Serum Albumin (BSA) adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:100:1852mm$^2$/ml, BSA conc.: 120ug/ml, pH 4.5 and temperature: 37°C).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Volumes</td>
<td>80.47 ± 0.51</td>
</tr>
<tr>
<td>Large Volumes</td>
<td>88.62 ± 0.75</td>
</tr>
</tbody>
</table>

5.4.6 BSA Alexa Fluor 488

Polystyrene microspheres were optimized to bind abundant amounts of purified BSA in the previous experiments. The spheres have an extremely high specific surface area and are hydrophobic to facilitate irreversible binding. Since the surface area may still be an underlining issue for the SMP system, a more sensitive detection method was tested against the microspheres. BSA conjugated to alexa fluor 488
was purchased as the study protein with the ability to be quantified through its fluorescent tag up to nanogram levels. The results show that the tagged surface interfered with adsorption significantly, as no protein was adsorbed onto the microspheres. The modified surface of the protein may hide specific interacting amino acid residues that were before interacting with the polymeric surface. The method was therefore not used in future SMP experiments.

Figure 41  BSA Alexa Fluor 488

The affect of BSA 488 adsorption onto polystyrene microspheres (surface area- to- volume ratio 1:100:1852mm²/ml, BSA conc.: 120µg/ml, pH 4.5 and temperature: 37°C).

Table 22  % Protein Bound Relative to Total BSA-488

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene Microspheres</td>
<td>-10.46 ± 15.01</td>
</tr>
</tbody>
</table>

5.5 Positive Control – Test Polystyrene Microspheres against Past Experiments

5.5.1 Experiments 1-7 Poly-L-Arginine

After the proper adsorption parameters were established to get ~90% binding, the positive control was used against previous experimental environments to justify if they were in fact, pro or anti-adsorption systems. The polystyrene beads were incubated in 44µg/ml of poly-L-arginine in PBS buffer for 1 hour at 37°C. Poly-L-arginine was proposed to discourage binding because of the lack of complimentary hydrophobic domains to facilitate irreversible binding. However, the positive control unveiled prevalent amounts of binding to its surface. Because the polystyrene spheres are a different polymeric system, adsorption mechanisms may not be completely translational. This data shows that our polymer could have been the anti-adsorption factor in the series of experiments with the amino acids.
Poly-L-arginine adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:100:1852mm²/ml, poly-L-arginine conc.: 44ug/ml, pH 7 and Temperature: 37°C).

Table 23 % Protein Bound Relative to Total Poly-L-Arginine

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 HR</td>
<td>91.79 ± 3.84</td>
</tr>
<tr>
<td>1 HR</td>
<td>79.77 ± 4.17</td>
</tr>
<tr>
<td>2 HR</td>
<td>96.07 ± 1.82</td>
</tr>
<tr>
<td>3 HR</td>
<td>93.63 ± 2.52</td>
</tr>
</tbody>
</table>

5.5.2 Experiments 8-11 Eosinophil Lysate

The exact system from the previous EO lysate experiments were tested with the polystyrene microspheres and the BCA assay was performed to quantify a total protein change. The BCA absorbance values in figure 43, at 562nm show little differences between each conditions, remembering that the “0” and “0+B” conditions do not contain EO lysate but are purely media and media + adsorbent conditions. This is an indication that FBS saturates the solution and inhibits EDGP adsorption, by masking the binding sites on the polymer surface before EDGPs can arrive at the polymer surface. An additional consideration is that RPMI, the media, is flushed with amino acids, vitamins, glucose, inorganic salts and substances that could consume binding sites as well, or create a large background in colorimetric assays such that the protein signal is hidden. In this experiment, the Bio-Rad protein assay was purposely chosen to have more leniencies with the buffer and to pick up more of a protein signal. As a result, it unveiled the media complications.
<table>
<thead>
<tr>
<th>Sample</th>
<th>No Dilution Absorbance (562nm)</th>
<th>1:10 Absorbance (562nm)</th>
<th>1:50 Absorbance (562nm)</th>
<th>1:100 Absorbance (562nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500k</td>
<td>2.115</td>
<td>1.854</td>
<td>1.04</td>
<td>0.796</td>
</tr>
<tr>
<td></td>
<td>2.12</td>
<td>1.776</td>
<td>0.984</td>
<td>0.703</td>
</tr>
<tr>
<td></td>
<td>2.152</td>
<td>1.661</td>
<td>0.989</td>
<td>0.752</td>
</tr>
<tr>
<td>500k+B</td>
<td>2.24</td>
<td>1.77</td>
<td>1.074</td>
<td>0.756</td>
</tr>
<tr>
<td></td>
<td>2.27</td>
<td>1.752</td>
<td>0.99</td>
<td>0.701</td>
</tr>
<tr>
<td></td>
<td>2.27</td>
<td>1.637</td>
<td>0.951</td>
<td>0.722</td>
</tr>
<tr>
<td>0</td>
<td>2.007</td>
<td>1.77</td>
<td>0.992</td>
<td>0.849</td>
</tr>
<tr>
<td></td>
<td>2.095</td>
<td>1.801</td>
<td>0.982</td>
<td>0.688</td>
</tr>
<tr>
<td></td>
<td>2.255</td>
<td>1.689</td>
<td>0.916</td>
<td>0.707</td>
</tr>
<tr>
<td>0+B</td>
<td>2.235</td>
<td>1.684</td>
<td>0.923</td>
<td>0.622</td>
</tr>
<tr>
<td></td>
<td>2.189</td>
<td>1.692</td>
<td>1.042</td>
<td>0.648</td>
</tr>
<tr>
<td></td>
<td>2.151</td>
<td>1.647</td>
<td>1.048</td>
<td>0.735</td>
</tr>
</tbody>
</table>

Figure 43 Experiments 8-11. Eosinophil Lysate

Bio-Rad protein assay results of EO Lysate adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:100:1852mm²/ml, EO lysate conc.: 500k/ml, pH 7 and Temperature: 37°C⁰).

5.5.3 Experiment 12 BSA and Ionic strength

Finally, polystyrene microspheres were incubated in low and high ionic strength solutions with BSA. The results illustrate how important ionic strength is to adsorption. In low ionic strength environments, 90% of BSA was bound to the microspheres. Adversely, a 10-fold increase in ionic strength leads to 0% adsorption. In the next experiments to follow, ionic strength will be highly considered and adjusted to 0.01M.
**Figure 44** Experiments 12. BSA and Ionic Strength

Ionic strength influence on BSA adsorption onto polystyrene microspheres (surface area-to-volume ratio 1:100:1852mm$^2$/ml, BSA conc.: 1mg/ml or 120ug/ml, pH 4.5 and Temperature: 37°C).

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>% Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Ionic Strength</td>
<td>90.97 ± 0.57</td>
</tr>
<tr>
<td>High Ionic Strength</td>
<td>-3.74 ± 0.14</td>
</tr>
</tbody>
</table>

### 5.6 SEM Adsorption

Since protein adsorption is a complex mechanism, the positive control provided guidance toward a working system and insight into why the previous experiments did not work. As such, the findings were implemented and adjusted on a new system to encourage protein binding. 2-sulfopropyl methacrylate (SEM) was copolymerized with tBA, PEGDMA and DMPA at different weight percents to leave the SMP samples with negative functional groups on their surfaces. Binding experiments were carried out with BSA at its isoelectric charge.

Results show that SEM polymers bind more protein per surface area than the other adsorbent samples. Polystyrene microspheres were used as a positive control but because of its highly concentrated state, the surface- to-volume ratio for the spheres was 1852mm$^2$/ml contrary to the other samples that were 262mm$^2$/ml. Thus, polystyrene sheets were purchased and sized as an additional control to eliminate surface area bias to binding and other variables introduced from free floating microspheres rather than a solid stationary adsorbent support. Nonetheless, binding per surface area was normalized as seen below.

From the polymer characterization data, the 2.5% SEM samples between batches possess differences that could ultimately influence binding. The surface of 2.5% from batch 1 has a larger dispersive (non polar) component than that of batch 2 shown in table 3. Though contact angle in air can be misrepresentative of
the same surface in aqueous solutions, the trend change in hydrophobicity exists with increasing wt % of SEM. Thus, the hydrophobicity could potentially translate to the number density of SEM methyl groups at the surface in lieu of their OH groups. If this is the case, the increase in polarity between adjacent binding sites of the polymer under aqueous solutions could lead to lateral repulsion upon protein binding and thus less binding. The differences in the rubbery modulus between batches in 2.5% SEM could also affect protein binding as cells bind to stiffer materials over softer materials in cell culture. The higher polar groups could alter the number of cross links formed during the polymerization processes by repulsion. Phase changes could exist within each polymer coupon. Nonetheless, figure 45 shows that the SEM SMPs dominate BSA adsorption over the other adsorbents, including polystyrene, which is known to adsorb protein readily and is in fact used to develop binding assays used for biomedical applications. Nylon is the base component of the Enterotest, which is used in preliminary clinical tests to capture biomarkers of EoE. The results below suggest that our system may bind protein better than nylon, and with development, has the propensity to bind substantial amounts of EDGPs. Small differences of negatively charged functional groups on the SMP surface can greatly affect protein adsorption. From 0% SEM to 2.5% SEM, there is >50% change in BSA adsorption. Figure 45 illustrates how hydrophobicity (polystyrene, gold SMP) or polarity alone (nylon) does not yield maximal BSA adsorption. The spacing of the functional groups relative to each other and the surface potential (electrostatic charge) along with hydrophobicity influence binding.
Figure 45 SEM Adsorption of BSA

Adsorbents from two different batches were tested for adsorption of BSA. Comparisons were made against polystyrene microspheres with *P< 0.01.
After the binding experiment, the SEM SMP samples were collected, washed and visualized for further evidence of protein bound to their surfaces. Figure 46 shows BSA bound to the polymer surface after its incubation step compared to a polymer incubated in media alone. Figure 47 shows a closer look of the polymer surfaces under a 10X magnification captured on an optical bench microscope. Visually and through the values obtained by the colorimetric assay, the saturation point of the polymers may be near the 1% SEM SMP sample as there is not much change between 1% and 2.5%. In addition, it should be noted that little SEM monomer was incorporated into the SMP system but drastic changes occurred between samples. The 0% SEM bound <10% of the total protein in solution where the mere 1% addition of the SEM monomer yielded >65% adsorption of BSA. In the previous experiments, CEA was incorporated at 5%, 10% and 15%, which could have over populated the polymer surface. The distances of the functional groups from each other are an important consideration in adsorption studies, as proteins interact not only with the polymer surface, but also with each other. If two proteins are binding to adjacent binding sites, repulsion can occur if they are too close to one another. This event is reduced by neutralizing the protein through the pH of the media but this only adjusts the net charge, such that repulsion is minimized but can still occur.

<table>
<thead>
<tr>
<th>Sample Code. Batch 1</th>
<th>% Protein Bound</th>
<th>Sample Code. Batch 2</th>
<th>% Protein Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microspheres</td>
<td>39.89</td>
<td>Microspheres</td>
<td>83.31</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>11.82</td>
<td>Polystyrene</td>
<td>11.18</td>
</tr>
<tr>
<td>Nylon</td>
<td>2.25</td>
<td>Nylon</td>
<td>2.72</td>
</tr>
<tr>
<td>Gold</td>
<td>6.10</td>
<td>0% SEM</td>
<td>7.26</td>
</tr>
<tr>
<td>0% SEM</td>
<td>9.44</td>
<td>0.5% SEM</td>
<td>18.77</td>
</tr>
<tr>
<td>0.5% SEM</td>
<td>29.32</td>
<td>1% SEM</td>
<td>71.47</td>
</tr>
<tr>
<td>1% SEM</td>
<td>65.62</td>
<td>2.5% SEM</td>
<td>78.73</td>
</tr>
<tr>
<td>2.5% SEM</td>
<td>61.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 46** SEM Polymer after 3 Hour Incubation

SEM polymer incubated in media alone (Left) compared to SEM polymer incubated in BSA (Right)
Figure 47 Images of the SEM Polymer after 3 Hour Incubation

BSA adsorption onto the SEM SMP imaged with an optical microscope at 10X magnification.
6. Conclusion

The results of the present study demonstrate that altering the surface chemistry of an acrylate-based SMP can encourage protein binding onto its surface with additional system adjustments. SEM SMPs had a higher yield of protein adsorption than known adsorbents used currently in biomedical applications such as polystyrene. Additionally, our 1% and 2.5% SEM polymers bound BSA at least 60% better than nylon, the base material of the esophageal string test with a known capacity to capture proteins and cells in-vivo. This suggests the SEM polymers have potential to improve current diagnostics of EoE by adsorbing substantial quantities of protein in a minimally invasive way with development. Further tests and optimization of the SEM SMP system for specific adsorption to biomarkers of EoE would increase the sensitivity of the eventual device.

During the developmental process, difficulties in improving the current diagnostics by depending purely on electrostatic and hydrophobic interactions for adsorption of EoE biomarkers onto our SMP became evident. The complexity of this aim originates from the vast collection of dominating factors for adsorption that compete with the surface chemistry of our biomaterial and the relatively weak non-covalent interactions we are basing specific adsorption on. The combination of surface chemistry and high surface area could strengthen these weak interactions and facilitate more adsorption.

Chemical modification of the SMP through copolymerization was performed rather than other surface alteration techniques such as texturing, ligand attachment or thin film deposition. Because of the eventual desire to adsorb proteins specifically, and the complexity that arises from processing, validation of the modified surface and additional multi-procedural stages associated with these other techniques, which would ultimately reside outside the time frame for this study, chemical modification was pursued and optimized.

Since EoE is a chronic inflammatory disease of the esophagus with a high rapport for misdiagnosis through the invasive procedure of endoscopy, the progression toward a better diagnostic for this disease is well justified. While the esophageal string test (“EST”) is a vast improvement to a minimally invasive diagnostic for this disease, the surface of the SEM polymer already binds substantially more protein than nylon, the base material of the EST. The reason for EST capturing protein in-vivo may be due to its large surface area. Together, with the surface chemistry of our functionalized SMP and the addition of
large surface area, our SMP system could be an even greater improvement to the diagnostics in place. Through the developmental stages of adsorption testing, electrostatic interactions were highly influenced by the media. Since these are the interactions we are depending on for specific interactions, optimization and more development of the polymer surface under physiological mimicking systems should be implemented. Since the SEM SMP was tested under a pH that matched the isoelectric point of BSA, the study protein, it would be beneficial to test if the SEM polymers adsorb cationic proteins of EoE at a pH of 7. This would unveil if the active functional groups on the surface of the polymer encourage specific binding with the electrostatic interactions in play on the protein surface. It was also found that competing proteins, ions and molecular species could bind preferentially to our polymer system and consume binding sites originally meant for the target protein. Additional optimization and tests could be performed to correct for this event. It may be that we do not need specific binding of the EDGP’s as long as they are captured at a high degree along with other esophageal proteins. If the surface area of our polymer system were increased substantially or specific texture or nano pockets were incorporated for capture of the EDGPs in combination of the tailored SEM SMP, the polymer could be corrected for competing proteins and molecules. The SEM polymer would consequently be tested under pH 7 with a mixture of proteins including EDGPs to find the optimal loading capacity of our polymer system.

The SEM SMP could also be valuable for in-vitro medical research. We have shown SEM SMP binds more effectively than polystyrene, a common polymer used in biomedical applications for attaching cells to the bottom of cell culture plates, affinity chromatography or immunoprecipitation to purify and study biological samples and microspheres used to optimize multiplex assays. Our SEM SMPs can pave the way for similar applications with development.

In summary, the results of the present study illustrate how the right balance of electrostatic and hydrophobic functional groups on the SMP could influence protein adsorption for the eventual development of a diagnostic for EoE. Through the experimental process, the dependencies of protein adsorption were discovered. The mechanism is based on weak, non-covalent interactions. As such, chemical modification of SMPs coupled with high surface area may provide better specificity strength and greater binding of the target proteins. In future work, the SMP can be altered for the capture of EDGPs through physical entrapment between micro-crevasses, which match the size of the target protein as
described briefly above. Another route could be incorporating active biological species into the polymer such as antibodies or amino acid peptide sequences to facilitate a specific response in-vivo.
REFERENCES


19. Lendlein, A. & Kelch, S. Shape-Memory Effect From permanent shape.


Appendix A: Detection

A.1 Overview of Detection Methods

Many protein quantification methods were considered in the initial stages of the experimental design, illustrated in tables 26-28. Based on these findings, ELISA’s and the micro BCA assay were mainly used because of their high sensitivity and reproducibility. ELISA’s were performed when quantifying one protein species from a pool of proteins in solution and the micro BCA assay was used when quantifying purified protein *.

Although there are various other methods for protein detection onto polymer surfaces such as Fourier transform-attenuated total reflection infrared spectroscopy (ATR-FTIR), ellipsometry, total internal reflection fluorescence spectroscopy (TIRF) or using radioisotope-labeled proteins, they were not implemented in the experimental design because of their availability and the limited time frame of this project9,13. Instead, UV absorption, fluorescence, colorimetric assays and antibody-based assays were utilized as our choices to measure protein concentrations. These choices are suitable for both elution or solution depletion methods but the critical determinants for these assays are their level of sensitivity because of our low polymeric surface area. At these low protein levels, the assay is required to be sensitive enough to discern low protein concentrations and have reproducible results at these small scales.

*The only exception was in the positive control experiment: “Experiments 8-11 Eosinophil Lysate,” where a Bio-Rad protein assay was used, analogous to the Bradford assay. Because of media interference with the micro BCA assay and the high costs associated with the multiple ELISAs, the Bio-Rad protein assay was substituted.
Table 26: Antibody-Based Assays.

<table>
<thead>
<tr>
<th>Antibody based assays</th>
<th>Sensitivity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Concentrations ≤ 1.0 pg/ml</td>
<td>$$$ Multi-step procedure (time expensive)</td>
</tr>
<tr>
<td>Western Blot- Mini Gels</td>
<td>Concentrations ≤ 100 pg</td>
<td>$$$ High variability from gel to gel Multi-step procedure (time expensive)</td>
</tr>
</tbody>
</table>

Antibody technology grants high specificity and sensitivity but can be expensive.
Table 27: Absorbance-Based Assays

<table>
<thead>
<tr>
<th>Absorbance based assays</th>
<th>Sensitivity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Plus (Bradford) micro-assay</td>
<td>1 µg/ml to 25 µg/ml</td>
<td>Proteins precipitate over time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High protein-to-protein signal variability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interference with detergent</td>
</tr>
<tr>
<td>Modified Lowry</td>
<td>1 µg/ml to 1.5 mg/ml</td>
<td>Protein to protein variation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lengthy, multistep procedure (additional variability)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interference with detergents, disulfides, copper chelating agents, carbohydrates, glycerol and other substances</td>
</tr>
<tr>
<td>Micro BCA method</td>
<td>0.5 µg/ml to 40 µg/ml</td>
<td>Not compatible with reducing agents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Susceptible to interference with thiols, copper chelating agents, tyrosine, cysteine, tryptophan and other substances</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein-to-protein variability</td>
</tr>
<tr>
<td>Absorbance at 280 nm</td>
<td>50 µg/ml to 2 mg/ml</td>
<td>High protein-to-protein variability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Detection influenced by nucleic acids and other contaminants</td>
</tr>
<tr>
<td>Nano-Drop A280</td>
<td>0.1 mg/ml to 100 mg/ml BSA</td>
<td>Quick</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reads aromatic amino acids</td>
</tr>
</tbody>
</table>

Colorimetric assays or absorbance of pure protein have a large linear range, are less time consuming and complex than antibody- based assays and are cost efficient. These are non specific assays where pure protein is usually detected.
Table 28: Fluorescence-Based Assays.

<table>
<thead>
<tr>
<th>Fluorescence based assays</th>
<th>Sensitivity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanoOrange® assay</td>
<td>10 ng/ml to 10 µg/ml</td>
<td>Samples can be read up to six hours later</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low protein-to-protein signal variability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Detection not influenced by reducing agents or nucleic acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensitive to salts and detergents</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toxicity levels unknown</td>
</tr>
<tr>
<td>CBQCA</td>
<td>10 ng/ml to 150 µg/ml</td>
<td>Multistep procedure (additional variability)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not compatible with buffers containing amines or thiols</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Highly toxic</td>
</tr>
<tr>
<td>Quant-iT™ Protein Assay Kit</td>
<td>0.25 µg/ml to 4 µg/ml</td>
<td>Samples can be read up to 3 hours later</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low sample volumes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Highly basic proteins behave aberrantly</td>
</tr>
<tr>
<td>Flamingo Fluorescent Gel Stain</td>
<td>Sensitivity limit of 0.25ng-5ng</td>
<td>Linear range over 3 orders of magnitude</td>
</tr>
<tr>
<td></td>
<td></td>
<td>UV and Mass Spectroscopy compatible</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multistep procedure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Variability from gel to gel with electrophoresis</td>
</tr>
</tbody>
</table>

Fluorescent assays can be as sensitive as antibody based assays but are non-specific (unless the protein of interest has a fluorescent tag itself). These are more cost efficient than antibody technology but can be toxic.
A.2 Micro BCA Assay

The micro BCA assay is a colorimetric assay with a bicinchoninic acid formulation developed to quantitate total protein\textsuperscript{32}. This assay was used in the project to quantitate pure protein after performing the binding experiments. Because our initial study protein was poly-L-arginine, a synthetic protein, the BCA assay was chosen because of its compatibility, sensitivity with low toxicity and good reproducibility for this protein. While the assay caters to the study protein, it is susceptible to reducing agents and other interfering substances that may exist in media solutions. As such, media solutions were tested to see if they elicited a false positive signal. The buffers initially chosen were Hanks balanced salt solution (HBSS) and RPMI because they both are used in cell culture to mimic physiological environments. These buffers were diluted with DI water and/or PBS and the BCA assay was performed. Tables 29 and 30 show the net absorbance values at 562nm. Results show that both buffers elicit a strong signal at the same absorbance used to detect protein that would ultimately interfere with the quantification of the protein and as such, cannot be used in the binding experiments. These false positive results decrease the sensitivity of the assay for the study protein and contribute to high background noise. BSA was diluted in PBS to generate a standard curve as shown in Table 31. The result of the standard curve clearly shows the absorbance values reside within the linear range of the assay, 2-40ug/ml, with little interference of the buffer which could otherwise hide the signal of the protein. The high end of the standard curve has an absorbance of approximately 0.7nm. For the Hank’s buffer without any protein present, a dilution between 1:8 and 1:16 would be necessary to be within the high end of this linear range and a dilution of about 1:32 with the RPMI buffer. These baseline dilutions would dilute out the protein to an undetectable level by this assay rendering it unpractical for our application. Dialysis, lyophilization or use of a rotary evaporator could be performed after the binding experiment to displace the interfering media but these processes are lengthy, and add both inter and intra experimental variability. As such, PBS or acetate buffer was used in place, with careful consideration of contents that may elicit a false positive signal.
**Table 29: BCA Assay with Hanks Balanced Salt Solution (HBSS)**

<table>
<thead>
<tr>
<th>Dilutions of HBSS</th>
<th>Net A(562nm) Repeat 1</th>
<th>Net A(562nm) Repeat 2</th>
<th>Net A(562nm) Repeat 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO DILUTION</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
</tr>
<tr>
<td>1:2</td>
<td>3.549</td>
<td>3.533</td>
<td>3.585</td>
</tr>
<tr>
<td>1:4</td>
<td>1.903</td>
<td>1.925</td>
<td>1.893</td>
</tr>
<tr>
<td>1:8</td>
<td>1.024</td>
<td>0.979</td>
<td>0.99</td>
</tr>
<tr>
<td>1:16</td>
<td>0.577</td>
<td>0.556</td>
<td>0.582</td>
</tr>
<tr>
<td>1:64</td>
<td>0.238</td>
<td>0.242</td>
<td>0.23</td>
</tr>
<tr>
<td>1:128</td>
<td>0.178</td>
<td>0.173</td>
<td>0.172</td>
</tr>
</tbody>
</table>

**Table 30: BCA Assay with RPMI Diluted with Water (W) or PBS (P)**

<table>
<thead>
<tr>
<th>Dilutions of RPMI</th>
<th>Net A(562nm) Repeat 1</th>
<th>Net A(562nm) Repeat 2</th>
<th>Net A(562nm) Repeat 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI: NO DILUTION</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
</tr>
<tr>
<td>1:2 W</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
</tr>
<tr>
<td>1:4 W</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
</tr>
<tr>
<td>1:8 W</td>
<td>2.633</td>
<td>2.449</td>
<td>2.512</td>
</tr>
<tr>
<td>1:16 W</td>
<td>1.446</td>
<td>1.388</td>
<td>1.393</td>
</tr>
<tr>
<td>1:32 W</td>
<td>0.811</td>
<td>0.795</td>
<td>0.796</td>
</tr>
<tr>
<td>1:64 W</td>
<td>0.461</td>
<td>0.45</td>
<td>0.453</td>
</tr>
<tr>
<td>1:128 W</td>
<td>0.284</td>
<td>0.276</td>
<td>0.332</td>
</tr>
<tr>
<td>1:256 W</td>
<td>0.153</td>
<td>0.143</td>
<td>0.164</td>
</tr>
<tr>
<td>1:2 P</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
</tr>
<tr>
<td>1:4 P</td>
<td>OVRFLW</td>
<td>OVRFLW</td>
<td>3.501</td>
</tr>
<tr>
<td>1:8 P</td>
<td>2.354</td>
<td>2.463</td>
<td>2.413</td>
</tr>
<tr>
<td>1:16 P</td>
<td>1.28</td>
<td>1.326</td>
<td>1.366</td>
</tr>
<tr>
<td>1:32 P</td>
<td>0.738</td>
<td>0.757</td>
<td>0.777</td>
</tr>
<tr>
<td>1:64 P</td>
<td>0.422</td>
<td>0.435</td>
<td>0.452</td>
</tr>
<tr>
<td>1:128 P</td>
<td>0.271</td>
<td>0.273</td>
<td>0.29</td>
</tr>
<tr>
<td>BSA concentration (ug/ml)</td>
<td>Net A(562nm)</td>
<td>Net A(562nm)</td>
<td>Net A(562nm)</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td></td>
<td>Repeat 1</td>
<td>Repeat 2</td>
<td>Repeat 3</td>
</tr>
<tr>
<td>200</td>
<td>2.78</td>
<td>2.61</td>
<td>2.739</td>
</tr>
<tr>
<td>40</td>
<td>0.746</td>
<td>0.751</td>
<td>0.767</td>
</tr>
<tr>
<td>20</td>
<td>0.423</td>
<td>0.421</td>
<td>0.424</td>
</tr>
<tr>
<td>10</td>
<td>0.252</td>
<td>0.238</td>
<td>0.234</td>
</tr>
<tr>
<td>5</td>
<td>0.146</td>
<td>0.15</td>
<td>0.148</td>
</tr>
<tr>
<td>2.5</td>
<td>0.122</td>
<td>0.121</td>
<td>0.121</td>
</tr>
<tr>
<td>1</td>
<td>0.116</td>
<td>0.117</td>
<td>0.117</td>
</tr>
<tr>
<td>0.5</td>
<td>0.115</td>
<td>0.111</td>
<td>0.113</td>
</tr>
<tr>
<td>0</td>
<td>0.113</td>
<td>0.088</td>
<td>0.124</td>
</tr>
</tbody>
</table>

Several adsorption experiments were performed but minimal amounts of binding occurred and the detection limit of the micro BCA assay was challenged. Figure 48 displays experiment 12 with BSA adsorbed onto CEA SMPs. Negative values and large error bars illustrate the amount of protein bound is within the noise of the assay. The solution depletion method was used as an indirect method to quantitate protein bound to the adsorbents (where the change in protein concentration was calculated) and requires the assay to discern similar values if minimal adsorption is taking place, thus the limit of detection is crucial. Exposing large surface areas of the polymer to the protein solution did not improve adsorption and so the detection method was reconsidered. If the polymers have monomers that did not completely polymerize, the free species may be released during the binding experiment into the media, and in this way, could alter the reaction of the colorimetric micro BCA assay. Since CEA is a reducing agent, further detection methods were tried to eliminate this possibility.
A.3 Bio-Rad Protein Assay

The Bio-Rad assay is a total protein assay based off the Bradford assay with a linear range of 1.2ug/ml to 10ug/ml using the micro assay procedure. This assay is less sensitive to reducing reagents and other interfering substances compared to the micro BCA assay. Therefore, it was used to measure protein from experiment 12. Results in figure 49 show the presence of negative values and large error bars even still. To eliminate the possibility of interference of a false positive or negative result altogether from media with free monomer species, a non-colorimetric assay was performed.

Figure 49 Bio-Rad Assay Performed on Experiment 12 CEA

The linear range is 1.2ug/ml-10ug/ml
A.4 Spectroscopy

Aromatic amino acids of proteins elicit an absorbance at 280nm. Even though this is a less sensitive method of detection, it was thought to be useful to try this method incase an adverse reaction was occurring between the colorimetric working reagents and the media solution encompassing small quantities of unpolymerized monomer content after a 3 hour incubation period. A spectroscopic reading at 280nm was used on the samples from experiment 12. Two micro liters of each sample was placed on the Biotek Take 3 micro-volume plate 16 times to account for error in the spectroscopy readings and pipetting. Figure 50 displays no binding. The large deviations show that we are still within the noise of the assay. Low amounts of binding may just be occurring. Since low sample volumes could account for some of this error, one more measurement was taken using higher sample volumes.

![BSA Adsorption: Spectroscopy with micro-volume plate](image)

**Figure 50** Spectroscopy Performed on Experiment 12 CEA

A biotek synergy machine was used with the Take 3 micro-volume plate to read protein levels at 280nm. 16 repeats per condition were performed. A280nm linear range is 0.1mg/ml-100mg/ml.

A sample volume of 100ul was placed in a microcuvette and measured by a biophotometer. Each sample was performed in triplicate. Figure 50 shows tighter standard deviations but negative values still persists. Oxidation and reduction reactions should not affect the results of this method as the protein samples are read without additions of other reagents and nucleic acids and aromatic amino acids are read. The buffer should not have any contaminants that would interfere even after incubation with the polymers. These results suggest the sensitivity of the assay needs to be addressed.
Figure 51  Spectroscopy Performed on Experiment 12 CEA

A biophotometer was used to read protein levels at 280nm. 3 repeats per condition were performed. A280nm linear range is 0.1mg/ml-100mg/ml.

A.5 Flamingo Fluorescent Gel Stain

Flamingo stain is a non specific or total protein fluorescent stain that is performed on SDS-PAGE gels\(^1\). Its linear range spans 3 orders of magnitude and its sensitivity limit approaches 0.25-5ng. This new detection method was used to see if we could remove ourselves from the noise of the assay and detect reproducible amounts of binding. A destaining step was carried out with 0.1% (w/v) Tween 20 for 10 minutes because the signal of the BSA bands was high. Figure 52 shows the BSA standard curves on each gel measured with a Typhoon fluorometer at 532nm. The first three lanes in each gel were over saturated and could not be used for quantification using densitometry. Since this stain is compatible with UV transilluminescence, the gels were exposed to UV and were quantified with densitometry using Quantity One software shown in figure 53. The linear range using this method is 960ng-60ng. Once densitometry was performed, the standard curve was generated shown in figure 54. The values from both gels per condition were averaged and plotted against the densitometry results. Because the standard deviations are extremely high, representing poor reproducibility between gels, the flamingo stain would not be a better detection method than the micro BCA assay for our binding experiments. The variability may be a contribution from the gels, the multistage procedure or loading error. Regardless, two gels should not be compared to one another because of such large differences.
**Figure 52** Flamingo Fluorescent Gel Stain on BSA Standards, Exposure with a Fluorometer at 532nm

The first three lanes were over saturated and could not be used for quantification.

**Figure 53** Flamingo Fluorescent Gel Stain on BSA Standards, Exposure with UV Transilluminescence

The BSA bands at 67kD were quantified using Quantity One software by densitometry. The linear range for these results is 960ng-60ng.
Standard curve was generated from averaging densitometry values of the two gels per condition and plotted against the known concentration. This detection approach is not suitable for our binding studies because of the large standard deviations, which is associated with poor reproducibility from gel to gel.

From these series of experiments, the micro BCA assay seems to be the best approach in terms of time, complexity, compatibility, sensitivity and reproducibility. Overall, these results suggest that the CEA SMP system does not bind above 2ug/ml of protein and that more system adjustments needed to be made to improve adsorption.

An additional method could be executed with the BCA assay to detect the protein directly on the solid support (polymer). This may help remove negative values from the graphs and can facilitate as a direct method for detection without elution. The polymer should be delicately washed and dabbed with a Kim wipe to rid of any collection of protein that is not bound onto the polymer system. Working reagent of the BCA assay could be added to the solid support until completely submerged. The incubation would proceed as normal. Afterwards, a small quantity of the reacted solution can be transferred to a micro plate and read at 562nm.

In future work, ATR-FTIR, ellipsometry, TIRF and radio labeling may be useful detection methods to consider in low surface area experiments. The direct detection methods may give a more accurate way to determine protein bound onto surfaces without an elution step to strip the protein from the polymer surface. Caution should be taken with radio labeled or fluorescently labeled protein as these changes to the study protein could influence adsorption. Labeling may alter the conformation of the true protein structure and ultimately create a false adsorption scenario. Protein structure of such alterations should be studied before going down this route. For instance, fluorescent tags can be hydrophobic, facilitating adsorption onto the hydrophobic surface or consume vital amino acid groups needed for adsorption.
Appendix B: Protocols

B.1 Acetate Buffer

Materials:

- Sodium acetate (Sigma)
- Acetic acid (reagent grade from Sigma)
- DI water passed through a 0.22um filter
- pH meter
- 70% ethanol
- 2 autoclaved glass beaker

Procedure:

1. Dilute 1N acetic acid to 0.1M with DI water.
2. Dilute 1N of sodium acetate to 0.1M with DI water.
3. Add 20mL of 0.1M acetic acid and 30mL of 0.1M sodium acetate to 950mL of DI H2O.
4. Mix the solution well before using the pH meter.
5. Place ethanol in one of the glass beakers and DI H2O in the other.
6. Place the pH meter probe in the ethanol to clean off particulate or debris and place in DI water before placing it in the acetate buffer.
7. Adjust the pH to 4.5 with 1N HCL or 1N NaOH.
B.2 Polystyrene Microspheres

Materials:

- Acetate buffer (I=0.01M, pH=4.45)
- 0.51µm polystyrene microspheres (Bang’s Laboratories)
- 1.5mL eppendorf tubes
- Pure BSA (Sigma-delipidated)
- 15mL polypropylene conical tubes
- Glass flow cytometer tubes (5mL)

Procedure:

1. Prepare 200µg/ml of BSA in acetate buffer to add to the protein conditions listed below
2. Label eppendorf tubes as follows (in bold):
   - Conditions performed in triplicate
     A. 120µg/ml (protein)
     B. 0µg/ml (acetate buffer)
     C. 120µg/ml + B (protein + microspheres)
     D. 0µg/ml + B (acetate buffer + microspheres)
   
3. Add 600ul of BSA prepared in step 1 to all protein conditions listed above (6 total tubes). In condition C, proteins will bind to the adsorbent. Condition A is the protein only sample where no adsorbent is added for a total protein measurement.
4. Add 600ul of acetate buffer to all other conditions. These conditions are to measure background noise.
5. Vortex the polystyrene microspheres for 30sec and dilute to a 0.1% solid (1:100 dilution) with acetate buffer. Note: To minimize pipetting error, larger serial dilutions of 1:10 (300ul spheres into 2.7mL acetate buffer) can be prepared for a final 1:100 dilution. This was performed in the glass, flow cytometer tubes.
6. Add 400ul of the diluted polystyrene microspheres to conditions C and D, pipette up and down three times to mix well.
7. Add 400ul of acetate buffer to conditions A and B to match the final 1ml volume to the other samples, pipette up and down three times to mix well.
8. Incubate samples at 37 °C in 5% CO2 incubator for 3hours.
9. Remove samples from the incubator and centrifuge for 15 minutes at 9300G force at 10°C.
10. Collect 700ul of supernatant being mindful not to perturb the microsphere pellet on the bottom of the eppendorf tube, and place in a separately labeled eppendorf.
11. Spin the supernatant collected in step 10 once more at 9300G force for 15 minutes at 10°C.
12. Collect 600ul of supernatant being mindful not to perturb the microsphere pellet on the bottom of the eppendorf tube, and place in a separately labeled eppendorf.
13. Store samples at -20°C until further use or continue with the micro BCA assay.
B.3 SEM Binding Experiment

Materials:

- 6 SMP samples (5x19x1.5mm²)
- 6 polystyrene samples (5x19x1.5mm²)
- 6 nylon samples (5x19x1.5mm²)
- Acetate buffer (I=0.01M, pH=4.45)
- DI H2O passed through a 0.2um filter with 1.2% SDS and 0.05% NaN3
- Pure BSA (Sigma-delipidated)
- 1.5mL eppendorf tubes
- 3 glass beakers (autoclaved)
- Tweezers
- 70% ethanol

Procedure:

1. Prepare 200ug/ml of BSA in acetate buffer to add to the protein conditions listed below
2. Label eppendorf tubes as follows (in bold):

   *Conditions performed in triplicate*
   
   A. 120ug/ml (protein) 
   B. 0ug/ml (acetate buffer) 
   C. 120ug/ml + B (protein + microspheres) 
   D. 0ug/ml + B (acetate buffer + microspheres) 
   E. 120ug/ml + 0% SMP (protein + 0%SMP) 
   F. 0ug/ml + 0% SMP (acetate buffer + 0% SMP) 
   G. 120ug/ml + 0.5% SMP (protein + 0.5%SMP) 
   H. 0ug/ml + 0.5% SMP (acetate buffer + 0.5% SMP) 
   I. 120ug/ml + 1% SMP (protein + 1%SMP) 
   J. 0ug/ml + 1% SMP (acetate buffer +1% SMP) 
   K. 120ug/ml + 2.5% SMP (protein + 2.5%SMP) 
   L. 0ug/ml + 2.5% SMP (acetate buffer + 2.5% SMP) 
   M. 120ug/ml + PS (protein + polystyrene) 
   N. 0ug/ml + PS (acetate buffer + polystyrene) 
   O. 120ug/ml + N (protein + nylon) 
   P. 0ug/ml + N (acetate buffer + nylon) 

3. Add 600ul of BSA prepared in step 1 to all protein conditions listed above. (24 total tubes) These will be the conditions where the proteins will bind to the adsorbents. The only exception is condition A, the protein only sample, where no adsorbent is added for a total protein measurement.
4. Add 600ul of acetate buffer to all other conditions. These conditions are to measure background noise.
5. Perform a 1:100 dilution of the DI H2O with 1.2%SDS and 0.05% NaN3 in acetate buffer. Note: Polystyrene microspheres are shipped in DI H2O with 1.2%SDS and 0.05% NaN3. To keep consistency between conditions, all are treated the same way. Thus, this solution is diluted in the same manner as the spheres.
6. Add 400ul of the final 1:100 DI H2O solution prepared in step 5 to all the conditions except the polystyrene microsphere conditions, C and D. This is because the microsphere conditions are already suspended in this solution.
7. Vortex the polystyrene microspheres for 30sec and dilute to a 0.1% solid (1:100 dilution) with acetate buffer. Note: To minimize pipetting error, larger serial dilutions of 1:10 (300ul spheres into 2.7mL acetate buffer) can be prepared for a final 1:100 dilution.
8. Add 400ul of the diluted polystyrene microspheres to conditions M and N.
9. With tweezers, add the other adsorbent samples in their appropriate eppendorf tubes being careful not to contaminate the tweezers. Make sure there is only one adsorbent per eppendorf tube and that it is fully submerged in the solution.
10. Incubate samples for 3 hours in a 37°C incubator with 5% CO2.
11. After the time point, remove all the samples from the incubator.
12. For all conditions EXCEPT the polystyrene microspheres, carefully and slowly aspirate 700ul of supernatant into a separately labeled eppendorf tube to measure the protein concentration. Do not touch the polymer with the pipette tip. Store at -20°C until further use or continue with the micro BCA assay.
13. For the polystyrene microsphere conditions, centrifuge the samples for 15 minutes at 9300G force at 10°C to pull down the spheres from the free protein solution.
14. Collect 700ul of the supernatant from the polystyrene samples being mindful not to perturb the microsphere pellet on the bottom of the eppendorf tube, and place in a separately labeled eppendorf.
15. Spin the supernatant collected in step 14 once more at 9300G force for 15 minutes at 10°C.
16. Collect 600ul of the supernatant from the polystyrene samples once more, being mindful not to perturb the microsphere pellet on the bottom of the eppendorf tube, and place in a separately labeled eppendorf. Store these samples at -20°C or continue with the micro BCA assay.
17. Once all the free protein in solution is collected for each sample, prepare a station to process the SMPs for microscopy.
18. Take three AUTOCLAVED glass containers and wash them thoroughly with DI water and ethanol. Suspend 70% ethanol in one of them. Suspend DI water in another. The beaker that is left will serve as a waste dispensary. Place a layer of Kim wipes on the bench to dab the polymer samples on.
19. Take CLEAN tweezers and soak them in the ethanol beaker for 10minutes.
20. Remove the tweezers from the ethanol and place in DI water to rinse.
21. Take a polymer sample and wash it with 1ml of PBS.
22. Dab the polymer sides on CLEAN Kim wipes and place the polymer sample in a 6well plate, keeping note which polymer it is.
23. Capture an image of the surface of the polymer under an optical microscope at 10X magnification and discard the polymer.
24. Repeat steps 19-23 for each polymer piece. The tweezers do not have to be submerged for 10min every time, just suspend in ethanol and rinse in DI water.
B.4 Eosinophil Lysate

Materials:

- 0.025M Sodium Acetate buffer (brought to pH 4.3 with Acetic Acid)
- 10X protease inhibitor cocktail: (Roche, Catalog #1 836 170): dissolve one tablet in 1mL of DI water and make appropriate aliquots to avoid freeze thaw cycles
- Sonicator with probe
- Ice and ice bucket
- 15mL, 50mL polypropylene conical tubes
- Purified blood eosinophils (EOs)

Note: Keep everything COLD (4°C) to attenuate de-granulation of EO’s

Procedure:

1. Calculate the volume of buffer needed to suspend the cells in for a final concentration of 5x10^6 cells/ml.
   Ex: For 10x10^6 Cells, 2mL of buffer is needed.
2. Prepare the volume calculated above of sodium acetate buffer with 10% protease inhibitor cocktail. Add 10X protease inhibitor cocktail at a 1:10 dilution into the appropriate amount of sodium acetate. Place the protease inhibitor cocktail in an ice bucket during use. Chill prepared buffer at 4°C before use.
3. Suspend the eosinophils in the sodium acetate buffer + PI solution made above at a final concentration of 5x10^6 cells/ml. Keep the suspension on ice.
4. Clean the sonicator before use by wiping down the probe with 70% ethanol and DI water.
5. Introduce the sonicator probe to the sample solution making certain the sample is placed on ice. Do not touch the edges or bottom of the tube with the probe.
6. Increase the amplitude knob such that the power is at 15% continuously for 10 seconds. Do not exceed 20% power- proteins may denature by the heat production. Also, do not lift the probe out of solution while the sonicator is on- this creates bubbles.
7. Turn the knob back to zero and allow the sample to chill on ice for 30sec to alleviate any heat generation.
8. Swirl the sample and repeat step 6 once more.
9. Centrifuge this lysate at 300g, 4°C for 10min to get rid of insoluble cellular contents.
10. Place the centrifuged sample in an ice bucket and gently collect the supernatant in a separate chilled 15mL conical avoiding the pellet on the bottom. The pellet retrieved after centrifugation can be thrown away.
11. Aliquot 110ul of lysate into separate eppendorf tubes with the concentration of the cells/volume ratio recorded (5x10^6 cells/ml).
12. Freeze samples at -80°C.