ASSEMBLY AND DYNAMIC BEHAVIOR OF MICROGEL THIN FILMS AND THEIR APPLICATION TO BIOINTERFACES

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Antoinette Bonhivert South

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ASSEMBLY AND DYNAMIC BEHAVIOR OF MICROGEL THIN FILMS AND THEIR APPLICATION TO BIOINTERFACES

Approved by:

Dr. L. Andrew Lyon, Advisor
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Jiri Janata
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Andrés García
Woodruff School of Mechanical Engineering
Georgia Institute of Technology

Dr. Laren Tolbert
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Niren Murthy
Wallace H. Coulter Department of Biomedical Engineering
Georgia Institute of Technology

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To my husband Clint, my mom, dad, and sister, and those who helped me throughout my journey
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There are a number of individuals to whom I owe enormous amounts of gratitude. First and foremost I would like to acknowledge my mom, dad, and sister Elise, who have been immensely encouraging during my entire life. Out of my parents, the person who has probably pushed me the most has always been my mom. Having a person in my life with such high expectations and who has always known that I could achieve great things has been a major driving force in all my accomplishments. I also owe my persistence, mindfulness, toughness, and creativity to my mom. My dad, who is quite possibly the complete opposite of my mother, yet also the complement to her, has also been very influential in my life. My dad is a leader, a motivator, and above all understands, cares for, and enjoys people, no matter who they are. My humor, humility, positive outlook, and ability to relate to others come from my dad. My little sister Elise has put up with me for her entire life, but I can’t imagine having any other sister except her. I am truly appreciative and happy to have a sister, and I hope she knows how special she is. I know we haven’t spent much time together more recently, but I hope our paths cross more often in the future. These three individuals have shaped me over my life into the open-minded and well-rounded person that I believe I am.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>xx</td>
</tr>
<tr>
<td>LIST OF SYMBOLS</td>
<td>xxi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xxii</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>xxiv</td>
</tr>
</tbody>
</table>

CHAPTER

1  Introduction 1
   1.1 Polymer Thin Films 1
   1.2 Hydrogels: Properties and Uses in Biological Applications 3
   1.3 Microgels 5
      1.3.1 Microgel Synthesis 5
      1.3.2 Characterization 6
         1.3.2.1 Atomic Force Microscopy 7
         1.3.2.2 Dynamic Light Scattering 10
      1.3.3 Microgels As Thin Film Building Blocks 11
   1.4 References 13

2  Host Response to Implanted Materials 19
   2.1 Introduction 19
   2.2 Non-Fouling Materials 21
   2.3 PEG-Modified Microgels 24
2.4 Outlook for Non-Fouling Microgels 27

2.5 References 28

3 Multi-Layered Non-Fouling Microgel Films 34

3.1 Introduction 34

3.2 Experimental 37

3.2.1 Materials 37

3.2.2 Microgel Synthesis 38

3.2.3 Substrate Functionalization and Microgel Film Assembly 39

3.2.4 Peptide Conjugation 41

3.2.5 Ligand Bioavailability Experiments 41

3.2.6 Atomic Force Microscopy 42

3.2.7 In Vitro Cellular Adhesion Studies 42

3.3 Results and Discussion 43

3.4 Conclusions and Outlook 57

3.5 References 58

4 Centrifugation as a Tool for Film Assembly 62

4.1 Introduction 62

4.2 Experimental 64

4.2.1 Materials 64

4.2.2 Microgel Synthesis 64

4.2.3 Microgel Characterization 65

4.2.4 Film Preparation 65

4.2.5 Multi-Layer Assembly 65

4.2.6 Atomic Force Microscopy Imaging and Analysis 66

4.3 Results and Discussion 66
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4 Conclusion</td>
<td>79</td>
</tr>
<tr>
<td>4.5 References</td>
<td>80</td>
</tr>
<tr>
<td>5 Self-Healing Multi-layered Films</td>
<td>85</td>
</tr>
<tr>
<td>5.1 Introduction to Self-Healing Materials</td>
<td>85</td>
</tr>
<tr>
<td>5.2 Experimental</td>
<td>87</td>
</tr>
<tr>
<td>5.2.1 Materials</td>
<td>87</td>
</tr>
<tr>
<td>5.2.2 Microgel Synthesis &amp; Characterization</td>
<td>87</td>
</tr>
<tr>
<td>5.2.3 Substrate Preparation &amp; Microgel Film Assembly</td>
<td>88</td>
</tr>
<tr>
<td>5.2.4 Film Characterization</td>
<td>89</td>
</tr>
<tr>
<td>5.2.5 Quantitative Stretching and “Break” Frequency Analysis</td>
<td>89</td>
</tr>
<tr>
<td>5.2.6 Film Thickness Measurements</td>
<td>92</td>
</tr>
<tr>
<td>5.3 Results and Discussion</td>
<td>92</td>
</tr>
<tr>
<td>5.4 Conclusion</td>
<td>121</td>
</tr>
<tr>
<td>5.5 References</td>
<td>123</td>
</tr>
<tr>
<td>6 Erosion of Substrate-Supported Degradable Microgels</td>
<td>127</td>
</tr>
<tr>
<td>6.1 Introduction</td>
<td>127</td>
</tr>
<tr>
<td>6.2 Experimental</td>
<td>129</td>
</tr>
<tr>
<td>6.2.1 Materials</td>
<td>129</td>
</tr>
<tr>
<td>6.2.2 DMHA Synthesis and Characterization</td>
<td>129</td>
</tr>
<tr>
<td>6.2.3 Microgel Synthesis</td>
<td>131</td>
</tr>
<tr>
<td>6.2.4 Microgel Characterization</td>
<td>131</td>
</tr>
<tr>
<td>6.2.5 Sample Preparation</td>
<td>131</td>
</tr>
<tr>
<td>6.2.6 Degradation Studies</td>
<td>132</td>
</tr>
<tr>
<td>6.2.7 Atomic Force Microscopy and Image Analysis</td>
<td>132</td>
</tr>
<tr>
<td>6.3 Results and Discussion</td>
<td>133</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>6.4 Conclusion</td>
<td>144</td>
</tr>
<tr>
<td>6.5 References</td>
<td>144</td>
</tr>
<tr>
<td>7 Outlook and Future Directions</td>
<td>147</td>
</tr>
<tr>
<td>7.1 Discussion</td>
<td>147</td>
</tr>
<tr>
<td>7.2 References</td>
<td>152</td>
</tr>
<tr>
<td>APPENDIX A: Non-Fouling Microgels Films on Implantable Substrates</td>
<td>154</td>
</tr>
<tr>
<td>A.1 Introduction</td>
<td>154</td>
</tr>
<tr>
<td>A.1.1 Breast Implants</td>
<td>155</td>
</tr>
<tr>
<td>A.1.2 Neural Electrodes</td>
<td>157</td>
</tr>
<tr>
<td>A.2 Experimental</td>
<td>158</td>
</tr>
<tr>
<td>A.2.1 Materials</td>
<td>158</td>
</tr>
<tr>
<td>A.2.2 Microgel Synthesis</td>
<td>158</td>
</tr>
<tr>
<td>A.2.3 Implant Surface Functionalization &amp; Microgel Film Assembly</td>
<td>159</td>
</tr>
<tr>
<td>A.2.3.1 Silicone Breast Implants</td>
<td>159</td>
</tr>
<tr>
<td>A.2.3.2 Silicon-Iridium Neural Electrodes</td>
<td>160</td>
</tr>
<tr>
<td>A.3 Results and Discussion</td>
<td>161</td>
</tr>
<tr>
<td>A.3.1 Breast Implants</td>
<td>161</td>
</tr>
<tr>
<td>A.3.2 Neural Electrodes</td>
<td>170</td>
</tr>
<tr>
<td>A.4 Conclusions &amp; Future Directions</td>
<td>172</td>
</tr>
<tr>
<td>A.4.1 Breast Implants</td>
<td>172</td>
</tr>
<tr>
<td>A.4.2 Neural Electrodes</td>
<td>173</td>
</tr>
<tr>
<td>A.5 References</td>
<td>173</td>
</tr>
<tr>
<td>VITA</td>
<td>178</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 4.1: Microgel Characteristics. [a] Determined by dynamic light scattering in pH 7.4 phosphate buffer containing 100 mM ionic strength (PBS) at room temperature. [b] Determined by atomic force microscopy on 30 minute passively deposited samples. [c] Determined by electrophoretic light scattering in PBS at room temperature. 68

Table A.1: XPS data showing atomic compositions from the neural probes surface. 171
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>AFM optical lever detection using a laser reflected from the back of a cantilever and directed to a photodiode detector. A cantilever is shown at two different deflection angles to illustrate the change that results on the detector.</td>
</tr>
<tr>
<td>1.2</td>
<td>Mosaic assembly using a variety of different microgels for a complex film.</td>
</tr>
<tr>
<td>2.1</td>
<td>Foreign body response to implanted materials. Note, images in figure are not drawn to scale.</td>
</tr>
<tr>
<td>2.2</td>
<td>PEG brushes (black) in low density (a) and high density (b), and their ability to resist adhesion of various proteins (colored).</td>
</tr>
<tr>
<td>3.1</td>
<td>Illustration of complex interface where non-specific protein adhesion is reduced and cellular adhesion peptide-containing microgels direct cellular adhesion.</td>
</tr>
<tr>
<td>3.2</td>
<td>Illustration of multi-layer film formation with atomic force microscopy images showing microgel monolayer (a), bilayer (b), three layers (c), and four layers (d).</td>
</tr>
<tr>
<td>3.3</td>
<td>Representative fluorescence microscopy images of fluorescently stained macrophages adhering on (a) tissue culture polystyrene (TCPS), (b) cleaned glass, and (c) 4 actively deposited layers of PEG cross-linked pNIPAm microgels. Scale bar represents 200 µm (a) and 100 µm (b &amp; c). (d) Quantification of cellular adhesion from fluorescence microscopy images with error bars representing the standard error.</td>
</tr>
<tr>
<td>3.4</td>
<td>Representative fluorescence microscopy images of fluorescently stained macrophages on four-layered non-fouling microgel films (a), four-layered non-fouling microgel films with RGD-microgels as fifth layer (b), four-layered non-fouling microgel films with RGE-microgels as fifth layer (c), cleaned glass (d), and tissue culture polystyrene (e). Scale bar represents 100 µm (a-d) and 200 µm (e).</td>
</tr>
<tr>
<td>3.5</td>
<td>Representative brightfield optical microscopy images of non-biotinylated core microgels (a), non-biotinylated core-shell microgels (b), biotinylated core microgels (c), and biotinylated core-shell microgels (d) after incubated with streptavidin coated beads for 24 hours at 4 °C. Scale bar = 5 µm.</td>
</tr>
<tr>
<td>3.6</td>
<td>Representative atomic force microscopy of assembled films containing four-layers of non-fouling microgels (a), four-layers of non-fouling microgels with RGD-conjugated core-shell microgels as fifth layer (b). Scale bar = 5 µm.</td>
</tr>
</tbody>
</table>
Figure 3.7: Fluorescence microscopy of fluorescently stained macrophages on four-layer non-fouling microgel film (a & f), four-layer non-fouling microgel film with RGD-conjugated core-shell microgels as fifth layer (b & g), RDG-conjugated core/shell microgels as fifth layer (c & h), cleaned plain glass (d & i), and TCPS (e & j). Amount of peptide conjugated was 1 mol % (b & c) or 10 mol % (g & h) of available acids on shell of core-shell microgels. Scale bars represent 100 µm for a-d and f-i and 200 µm for e & j.

Figure 3.8: Four-layer non-fouling microgel films after assembly (a) and after incubation in high ionic strength (100mM) phosphate buffered saline (b). Scale bars = 20 µm for large image and 5 µm for insets.

Figure 4.1: Atomic force microscopy of microgels (1) (a and b) and (2) (c and d) under passive deposition (a and c) and active centrifugal deposition (b and d). Inset scale bars are 0.5 µm (a and b) and 1 µm (c and d). On the right, radial distribution functions are shown for each particle type and deposition condition, illustrating the quantitative differences in particle spacing between active and passive deposition.

Figure 4.2: Microgel footprint area as a function of deposition time for centrifugation (active) and passive deposition techniques. (a) Microgel (1) and (b) microgel (2). *Denotes formation of a full monolayer. Error bars represent the standard error of the mean particle footprint area taken from three 10 µm x 10 µm (a) or 20 µm x 20 µm for (b) atomic force microscopy images.

Figure 4.3: Microgel (1) deposited over time passively for 30min (a & b), 16 hrs PA (c & d), centrifuged for 30 sec (e & f), centrifuged for 2 min (g & h), and centrifuged for 10 min (i & j).

Figure 4.4: Microgel (2) deposited over time passively for 30min (a & b), 16 hrs PA (c & d), centrifuged for 30 sec (e & f), centrifuged for 2 min (g & h), and centrifuged for 10 min (i & j).

Figure 4.5: Illustration showing relative size of AFM tip to microgel film sample, and how the real sample compares to the data output from the AFM scan.

Figure 4.6: Atomic force microscopy characterization of the two-step deposition process for microgel (2). (a) A sub-monolayer was passively deposited first and then subjected to centrifugal deposition. (b) A full monolayer was first deposited passively and then subjected to centrifugal deposition. (c) Shows a full monolayer deposited actively for comparison. Scale bar is 1 µm. (d) Bar graph showing the average particle footprint area for all samples. Error bars represent the standard error of the mean particle footprint area taken from three 20 µm x 20 µm AFM images.

Figure 4.7: AFM illustrating the affect of different ionic strengths on film assembly comparing the two deposition methods.
Figure 4.8: AFM images of microgel thin films constructed in a multi-layered fashion (using microgel 2). Each image has a 20 µm x 20 µm scan size with a 5 µm x 5 µm inset. Images were obtained from 1, 2, 3, and 4 layer films (left to right) formed by active deposition.

Figure 5.1: Stretching apparatus containing a translational stage and knob (a) that moves the left side of the setup (b) in the horizontal direction when the knob is turned. The other side of the apparatus is fixed (c). The sample is suspended in the air and sandwiched between two glass slides on either side (d).

Figure 5.2: Visualization of damage introduced by multiple “stabs” with a 5 µL pipette tip. Images were taken by (a, d, g) digital camera (scale bar = 2.5 mm), (b, e, h) brightfield optical microscopy (scale bar = 20 µm), and (c, f, i) atomic force microscopy (scale bar = 10 µm), before damage (a, b, c), after damage (d, e, f), and after healing by rehydration (g, h, i).

Figure 5.3: AFM of a four-layer carboxylated polystyrene microsphere film assembled with 400-500K PDADMAC (rigid sphere film) (a). Bright field transmission microscopy of the rigid sphere film after assembly (b & c), poked with a pipette tip (d & e), immersed in water (f), and dried (g). Red arrows in (f) denote desorbed particles. Scale bars represent 80 µm (b, d, f & g) and 20 µm (c & e).

Figure 5.4: Film damage introduced by (a) stretching and (b) bending deformation, as observed by brightfield optical microscopy (scale bar = 10 µm). Samples are shown after deformation (a, c) and after healing (b, d). During bending of the sample (c), the microgel film is present on the outer surface. Insets are 5 µm x 5 µm atomic force microscopy scans of the damaged or healed regions (scale bar = 1 µm).

Figure 5.5: PDMS that has been HCl-treated and APTMS-functionalized before (a) and after (b) stretching. Four microgel multilayers on HCl-treated and APTMS-functionalized PDMS before (c) and after (d) stretching.

Figure 5.6: AFM scans (20 µm x 20 µm) of defects induced on the same sample before deformation and after healing in order from left to right (scale bar = 5 µm) (a). AFM line profiles of each scan drawn across the entire image are shown to illustrate the reversibility of the phenomenon (b). The border color for each scan in (a) corresponds to the color of each curve in (b).

Figure 5.7: AFM of four-layered microgel films using BIS cross-linked pNIPAM-co-AAc microgels and 400-500K PDADMAC (a - c) and 100-200K PDADMAC (d - f). Films were interrogated after assembly (a & d), after stretching by 10% in length (b & e), and after healing with water (c & f).
Figure 5.8: Atomic force microscopy height images of a microgel monolayer (a), multi-layered films containing four microgel layers with different Mw PDADMAC 400-500K (b), 100-200K (c), and 40K (d) assembled on functionalized PDMS. Scale bar represents 2 µm.

Figure 5.9: Representative images from brightfield transmission optical microscopy of microgel multi-layered films containing four microgel layers with PDADMAC 400-500K (a), 100-200K (b), and 40K (c). Scale bar represents 20 µm.

Figure 5.10: Dependence of polycation molecular weight and number of microgel layers on the “break” frequency in microgel films.

Figure 5.11: Cartoon illustrating defect-induced stretching in materials before (a), and after at strains that cause microvoids (b) followed by crack-propagation (c). Each brightfield optical microscopy to the right shows this possible phenomenon in four-layered microgel films assembled in 400-500K PDADMAC before stretching (d), at 5.5% stretch strain (e) and at 11% stretch strain (f). Scale bar represents 10 µm.

Figure 5.12: Film thickness measurements taken before (a) and after “aging” in PBS of 100 mM ionic strength (b).

Figure 5.13: Bright field transmission microscopy of four-layered polystyrene spheres assembled with 400-500K PDADMAC. After assembly (a), stretched by 1 mm (10% of length) (b), stretched by 5 mm (50% of length) (c & d), after water addition (e & f) and dried (g & h). Scale bar represents 80 µm (a – c, e & g) and 20 µm (d, f, h).

Figure 5.14: Bright field transmission microscopy of damage in a four-layer film using BIS cross-linked pNIPAm-co-AAc and 400-500K PDADMAC. Films were bent with the film on the outside (a) and bent with the film inside (b). Scale bar represents 20 µm. AFM image was included from (b) to show more detailed morphological changes in the film (c).

Figure 5.15: Fluorescence microscopy of four-layered AFA-labeled microgels using 400-500K PDADMAC. View is from underneath the film. Images represent a monolayer (a), four-layer film (b), multi-layer stretched by 1 mm (11% of length) (c), another area where the multi-layer film was scratched (d), multi-layered film healed after water solvation and dried (e). Scale bar represents 20 µm.

Figure 5.16: Fluorescence microscopy of four-layered AFA-labeled microgels using 400-500K PDADMAC. Samples were placed upside-down with the microgel film facing down against the glass slide for imaging. Images represent a multi-layer film that was stretched by 1 mm (11% of length) (a & c), and healed after water solvation and dried (b & d). Scale bar represents 20 µm (a & b) and 8 µm (c & d).
Figure 5.17: Fluorescence microscopy of four-layered AFA-labeled microgels using 400-500K PDADMAC. Samples were placed upside-down for imaging. The 100x objective was used to photobleach a spot, and the film was interrogated using the 40x objective (a). The film was stretched by 1mm (11% of length) (b), and then healed (c). Stretched and healed five times (d). Scale bar represents 20 µm. Note: the gain was adjusted for (c) and (d) after obtaining (a) and (b).116

Figure 5.18: Fluorescence microscopy using a new sample of four-layered AFA-labeled microgels assembled with 400-500K PDADMAC. Samples were placed upside-down with the microgel film facing down against the glass slide for imaging. Each image represents a four-layered film after assembly (a & f), a photobleached spot that was burned in 40x objective (b & g), film stretched by 11% of length (c & h), film healed upon water solvation and dried (d & i), film stretched by 11% of length, water healed, and dried five times (e & j). Scale bars represent 80 µm (a – e) and 20 µm (f – j). Note: the “gain” was not changed for any of these images. 119

Figure 5.19: Brightfield transmission microscopy of stretched (a) and healed (b) AFA-labeled microgel multi-layered (four layers) films. Scale bar represents 20 µm.119

Figure 5.20: Fluorescence microscopy of four-layered AFA-labeled microgel polyelectrolyte films assembled with 400-500K PDADMAC. The photobleached spot was made under 40x magnification and interrogated using the 10x (plus 1.5 slider) objective (a). The spot was observed after 24 h in air (b) and after a 3 minute incubation in water and dried (c). Scale bar represents 80 µm.120

Figure 5.21: AFM of AFA-conjugated microgels assembled in a four-layer film using 400-500K PDADMAC before (a & c) and after (b & d) stretching by 11% in length and healed 10 times. Scale bars represent 5 µm (a & b) and 2 µm (c & d).121

Figure 6.1: Size distribution of degradable microgel hydrodynamic radii (R_h) determined by DLS (cumulants fit) at pH 3 (filled circles) and pH 7.4 (open circles) (a), and temperature-dependent microgel scattering intensity in pH 3 buffer (b).134

Figure 6.2: Three-dimensional renderings of AFM images obtained from a single microgel during erosion at 0 h (a, d), 24 h (b, e), and 434 h (c, f). Images were taken under dehydrated (a-c) or hydrated (d-f) conditions. 136

Figure 6.3: AFM two-dimensional image of an ensemble of eroded microgels, after 434 h in serum at 37 ºC, illustrating the “halo” effect around a dense polymer center for all particles. 137

Figure 6.4: Two-dimensional AFM height images of an ensemble of degradable microgels at time 0 (a, d), 24 h (b, e), and 434 h (c, f) scanned under dehydrated (a-c) and hydrated (d-f) conditions. 138
Figure 6.5: Average microgel height over time under dry imaging conditions. Data represents microgel erosion in serum at 37 °C (filled red circles) and room temperature (filled blue circles), and in pH 3 control buffer at 37 °C (open red circles) and room temperature (open blue circles). Error bars represent the standard deviation of the average particle height measured over three separately scanned images.

Figure 6.6: Two-dimensional AFM height images, scanned under dehydrated conditions, showing time 0 (a), and 481 h in pH 3 at room temperature (b) and 434 h in pH 3 at 37 ºC (c).

Figure 6.7: AFM height line profiles taken from an ensemble of hydrated microgels at different time points in serum at (a) 37 ºC and (b) room temperature.

Figure 6.8: AFM height line profiles taken from an ensemble of hydrated microgels at different time points in pH 3 control buffer at 37 ºC (a) and room temperature (b).

Figure 6.9: Two-dimensional height images of microgel erosion at room temperature imaged under dry conditions (a, b) and in liquid (c, d). Time points of time 0 (a, c) and 6 hours in serum (b, d) are shown. Scale bar represents 250 nm.

Figure A.1: AFM height images of hexane-cleaned silicone (a), and a microgel monolayer on cleaned silicone (b & c). Scale bar represents 5 µm.

Figure A.2: AFM height image (a) and phase image (b) of a microgel monolayer on functionalized silicone. Two representative height images of four microgel multi-layers (c & d) assembled on top the monolayer shown in (a & b). Scale bar represents 5 µm.

Figure A.3: AFM height images of microgel multi-layers after incubation in 70 % aqueous ethanol for 24 hours, followed by incubation in 10 % serum supplemented in 1X PBS at 37 ºC for 24 hours (a & b). Cleaned silicone (c) for comparison. Note that (b) and (c) are on the same z-scale. Scale bars represent 5 µm (a) and 2 µm (b & c).

Figure A.4: AFM height images of microgel multi-layers after taped to slide (a), pressed down upon (b), rubbed vigorously (c), incubation in water while attempting to interrogate the film via in liquid AFM (d), and after the “tape test” (e). Scale bar represents 5 µm.

Figure A.5: Transmission optical microscopy of microgel coated breast implants (a & d) and uncoated breast implants (b & e) that were explanted from swine after 1 month of implantation. A coated implant piece that was never implanted was included for comparison (c & f). Scale bars represent 10 µm (a-c) and 5 µm (d-f).
Figure A.6: AFM height images of an extracted microgel coated breast implant sample (a & d) and extracted uncoated implant sample (b & e) after 1 month of implantation. A microgel coated non-implanted piece was included for comparison (c & f). Note that a-c and d & f are on the same z-scale.  

168

Figure A.7: AFM height images of 2 month explantations: microgel-coated implant (a & b), uncoated implant (c & d), and uncoated implant that was not subject to the same sterilization procedure and taken straight out of the box from the manufacturer (e). Scale bar represents 5 µm.  

168

Figure A.8: AFM height profile scan of a neural probe taken straight from the manufacturer’s box. Scale bar represents 5 µm.  

171

Figure A.9: AFM height profile (a) and amplitude profile in region containing a raised iridium channel in the left part of the image (b) of four microgel multi-layers on cleaned and functionalized neural probes.  

172
# LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Illustration of complex interface where non-specific protein adhesion is reduced and cellular adhesion peptide-containing microgels direct cellular adhesion.</td>
<td>35</td>
</tr>
<tr>
<td>3.2</td>
<td>Illustration of inability to deposit microgels in physiologically-relevant buffer (pH 7.4) onto acrylic acid functionalized PET (a), and modification that enabled assembly (b).</td>
<td>45</td>
</tr>
<tr>
<td>6.1</td>
<td>Erosion of DMHA Cross-Linked Microgels</td>
<td>134</td>
</tr>
<tr>
<td>7.1</td>
<td>Direction of wrinkling due to buckling instability of a bilayer film containing two materials of different mechanical strength (a) and direction of film features in the microgel film from stretching (b) when stretched in the same direction as (a).</td>
<td>151</td>
</tr>
</tbody>
</table>
## LIST OF SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g(\tau) )</td>
<td>Autocorrelation function</td>
</tr>
<tr>
<td>( \tau )</td>
<td>Time interval</td>
</tr>
<tr>
<td>( \kappa )</td>
<td>Time constant</td>
</tr>
<tr>
<td>( D )</td>
<td>Diffusion constant</td>
</tr>
<tr>
<td>( q )</td>
<td>Diffusion wave vector</td>
</tr>
<tr>
<td>( k_B )</td>
<td>Boltzmann’s Constant</td>
</tr>
<tr>
<td>( T )</td>
<td>Temperature</td>
</tr>
<tr>
<td>( \eta )</td>
<td>Viscosity</td>
</tr>
<tr>
<td>( R_h )</td>
<td>Hydrodynamic Radius</td>
</tr>
<tr>
<td>( g )</td>
<td>Relative centrifugal force unit (gravity)</td>
</tr>
<tr>
<td>( R_s )</td>
<td>Microgel surface footprint radius</td>
</tr>
<tr>
<td>( \sigma )</td>
<td>Mechanical stress</td>
</tr>
<tr>
<td>( F )</td>
<td>Force</td>
</tr>
<tr>
<td>( A )</td>
<td>Cross-sectional area</td>
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<tr>
<td>( k )</td>
<td>Spring constant</td>
</tr>
<tr>
<td>( x )</td>
<td>Lateral displacement</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Langmuir-Blodgett</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-Assembled Monolayer</td>
</tr>
<tr>
<td>LbL</td>
<td>Layer by Layer</td>
</tr>
<tr>
<td>NIPAM</td>
<td>$N$-isopropylacrylamide</td>
</tr>
<tr>
<td>NIPMAm</td>
<td>$N$-isopropylmethacrylamide</td>
</tr>
<tr>
<td>LCST</td>
<td>Lower Critical Solution Temperature</td>
</tr>
<tr>
<td>VPTT</td>
<td>Volume Phase Transition Temperature</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy (Microscope)</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BIS</td>
<td>$N,N'$-Methylene bisacrylamide</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>RGD</td>
<td>Cellular Adhesion Peptide Sequence: Arginine, Glycine, Aspartic Acid</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>AAc</td>
<td>Acrylic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>APTMS</td>
<td>3-aminopropyltrimethoxysilane</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>EDC</td>
<td>N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>PDADMAC</td>
<td>Poly(diallyldimethylammonium chloride)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine) hydrochloride</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue Culture Polystyrene</td>
</tr>
<tr>
<td>PD</td>
<td>Polydispersity</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>AFA</td>
<td>4-acrylamidofluorescein</td>
</tr>
<tr>
<td>PSD</td>
<td>Power Spectra Density</td>
</tr>
<tr>
<td>DMHA</td>
<td>$N,O$-dimethacryloyl hydroxylamine</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus Amebocyte Lysate</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
</tr>
</tbody>
</table>
SUMMARY

Hydrogels, which are polymeric cross-linked networks that swell in aqueous environments, are versatile materials that can contain a variety of chemical functionalities, mechanical properties, and topographical features. Microgels are the stable colloidal form of hydrogel materials that range in size from approximately 100 nm to a few microns in diameter. While they also can exhibit similar properties to those of macrogels, microgels can be used as building blocks in a bottom-up approach to assemble films of higher complexity. In this dissertation, work is focused on understanding the assembly and behavior of microgel thin films as non-fouling surfaces, centrifugally deposited materials, self-healing coatings, and degradable constructs. Non-fouling films were assembled using PEG cross-linked microgels to reduce non-specific protein adsorption and mitigate cellular adhesion. These constructs were assembled in a polyelectrolyte multi-layered fashion, of alternating anionic microgels and cationic linear polymer, to effectively block the substrate from the biological environment and consequently exhibited control over cellular adhesion with the surface (Chapter 3). The utility and application of these non-fouling microgel coatings on functional implants was also explored (Appendix A). Centrifugal deposition was used to rapidly generate non-fouling microgel multi-layered interfaces on planar surfaces, and upon closer inspection of the microgel monolayers, it was found that the centrifugally deposited films contained closer-packed microgel assemblies with microgels of smaller footprint size, compared to microgels that are passively adsorbed to the surface (Chapter 4). Microgels that are centrifugally deposited may adopt a higher energy chain conformation than passively
adsorbed microgels, and this higher energy chain conformation may translate into the multi-layered materials. Nonetheless, the centrifugally deposited non-fouling microgel multi-layered films were found to effectively block macrophage adhesion (Chapter 3). Films were also assembled in a polyelectrolyte fashion on soft substrates, and were observed to become significantly damaged under mechanical manipulation (poking, bending, or stretching), but then self-heal upon addition of water (Chapter 5). By altering the building blocks of the polyelectrolyte multi-layered films, such as the molecular weight of the polycation between microgel layers or by using anionic rigid spheres as the particle in the assembly, changes in the observed film damage suggest that particle-linear polymer interpenetration and polyvalency likely play an important role in the strength and integrity of the microgel thin films. Fluorescently-labeled microgels were also used to interrogate how the films reorganize in the lateral direction (Chapter 5), and these early studies suggest that the microgel multi-layered films reorganize when damaged and also possibly when they are undamaged and simply incubated in an aqueous environment. Additional studies were also conducted on microgels synthesized with a hydrolyzable cross-linker, and by supporting these degradable constructs on substrates, detailed single-particle morphological changes during erosion could be interrogated in complex media such as serum (Chapter 6). This work, as a collection, demonstrates the ability to obtain information about microgel thin film assemblies and their behavior using microscopy techniques such as ambient and in liquid atomic force microscopy, brightfield optical microscopy, and fluorescence microscopy. The observations made here illustrate how microgels can be used to fabrication thin films that can be utilized in biological applications (non-fouling, self-healing, and erodable...
constructs), and how different deposition methods (centrifugal deposition and polyelectrolyte multi-layers) can dictate their behavior.
CHAPTER 1
INTRODUCTION

1.1 Polymeric Thin Films

For hundreds of years, the design and application of coatings, or films, have been of enormous interest. Coatings are involved in numerous applications, such as paints, photography, paper, medical devices, water repellants, adhesives, and cosmetics, to name a few. Consequently, there has been an enormous amount of research dedicated to understanding the assembly and behavior of films that serve as an interface between an underlying material and the surrounding environment. These films can be constructed from metals, inorganics (ceramics), or polymers, depending on their intended function. Polymers in particular have a wide range of available chemical and physical properties, and are therefore useful for making films that can satisfy a variety of conditions. For example, polymers can be used to make extremely hydrophobic surfaces, which is dependent on chemical composition and topographical features.\(^1\) Polymers can also be useful for protecting metals from corrosion, such as by providing a reservoir for an imbedded corrosion inhibitor.\(^2\) Polymeric thin films are also very useful as drug-eluting materials for controlled drug delivery.\(^3\) Much of the thin film work involving polymers has also found its inspiration from biology, and has sought to mimic these complex biological systems to gain insight as to how they function and how they can exploited in interesting and useful ways. Examples of polymeric coatings that are biology-inspired include the vascular system for self-healing materials,\(^4\) adhesive proteins from mussels for polydopamine-based adhesives,\(^5\) the micron-sized waxy papillae of the lotus leaf for superhydrophobic materials,\(^6\) or the extracellular matrix for tissue scaffolds.\(^7\)

There are many approaches to coating substrates with polymers. In general, polymers can either be grown, directly deposited, or self-assembled onto a surface.
Polymer film growth involves surface initiated polymerization, such as brush formation, grafting, or vapor deposition polymerization. Films that are physically deposited use techniques such as spin-coating or painting, which are mainly dictated by solvent evaporation. However, there has been a great deal of interest in the self-assembly of polymeric thin films, which typically offers better control of film thickness and also enables the study of interactions between the coating material, substrate, and solvent. Self-assembly methods include Langmuir-Blodgett (LB), self-assembled monolayer (SAM), or Layer by Layer (LbL). The LB approach, which is probably the oldest self-assembly film deposition technique, was first discussed by Katharine Blodgett and Irvin Langmuir using fatty acids. When amphiphilic molecules are added to water, and their concentration is below the critical micellar concentration (CMC), the molecules assemble at the water-air interface to reduce their surface energy. Virtually any type of substrate can then be coated by these amphiphilic monolayers by dipping it through the water-air interface, and it can be performed repeatedly to generate multi-layered films. To ensure the best coverage, the surface pressure of the monolayer at the air-water interface must be high enough so that there is adequate cohesion to survive transfer to the substrate.

Besides fatty acids, the LB approach has also been useful in depositing polymeric films, such as conductive polymers for thin film transistors. However, LB films suffer from film instability and lower quality of coverage, and therefore are not sufficient for long-term coatings that can be maintained in many environments. SAMs are also commonly used for film assembly, and the first study, involving surfactant SAMs assembled on metals, dates back to 1946. Molecules are chemisorbed from solution onto substrates due to an affinity of a portion of the molecule for the particular surface, and their assembly into monolayers minimizes the surface energy of the substrate. The assembled structure is close-packed and well-ordered, thus forming stable configurations on surfaces. As a result, SAMs are typically stronger than LB films. Alkane-thiols assembled on gold have become one of the most widely studied SAMs because of the
strong affinity between sulfur and transition metals. The chemisorption of thiols on gold has been used in the assembly of polymeric thin film, such as the study by Prime et al. for oligo(ethylene glycol) capped alkane thiols for controlled protein adsorption. However, while SAMs are usually more robust than LB films, they do suffer from instability in some environments, especially in vivo, and are limited to certain types of molecules. LbL, first established by Decher only a few decades ago, is a more general film fabrication technique that results in strong polymeric films. LbL involves the alternating deposition of oppositely charged polyelectrolytes, which typically leads to step-wise single-layer addition due to the self-regulation of polymers that repel each other during the deposition of one layer, and the drive for charge neutrality by the opposite charged polymer that is deposited in the next layer. Instead of simply reaching charge neutrality, however, each layer is typically added in excess and therefore there is a charge reversal that allows the next layer to be added. Additionally, complete charge neutrality is difficult to achieve because it is entropically unfavorable for the polymer chains to lose their mobility through the Coulombic attraction between opposite charges. Polymeric thin films that are assembled in LbL fashion are strong due to the polyvalent interactions and “fuzzy” structures from the molecular flexibility. As will be seen later in this dissertation, LbL assembly is a film fabrication method that led to both enabling and interesting characteristics of hydrogel films.

1.2 Hydrogels: Properties and Uses in Biological Applications

Hydrogels, which are cross-linked polymer networks that swell considerably in water, have been a topic of growing interest over the past twenty years due to their unique properties and the wide variety of applications in which they can be exploited. Hydrogels are particularly well suited for interfacing with biology because they consist mainly of water, can contain biological molecules either covalently or passively within their network, can be tuned in terms of their mechanical properties, and are able to
respond to external triggers such as changes in temperature, pH, small molecules, proteins, light, or electrical fields. It is this last characteristic, or responsivity, that enables hydrogels to exhibit complex and active interactions with biology, which is often needed to effectively control biological environments. For example, the thermoresponsive polymer poly(N-isopropylacrylamide), or pNIPAm, has been widely studied in hydrogel systems because a thermal trigger event induces the gel to phase separate from aqueous solution. In addition, it is worth noting here that pNIPAm is the main polymer used throughout this dissertation, with the exception of Chapter 6 where a related monomer poly(N-isopropylmethacrylamide) (pNIPMAm) is used. When pNIPAm by itself is a random coil in solution, hydrogen bonding occurs between the amide side chains and water, while the isopropyl group induces the hydrophobic ordering of water. As the temperature increases and reaches the lower critical solution temperature (LCST) (which is approximately 31 °C and 41 °C for pNIPAm and pNIPMAm, respectively), instead of the polymer-solvent interactions dominating the system, the polymer-polymer hydrophobic interactions become stronger and induce polymer phase separation. During this phase separation, the hydrogen bonds break between water and pNIPAm, thus releasing water from the polymer, and the pNIPAm takes on a globular polymer conformation. It is this favorable increase in the entropy of water that drives the thermal phase transition of pNIPAm in water. When pNIPAm is incorporated within a hydrogel network with cross-links, these gels also exhibit a phase transition that is near the LCST of pNIPAm. This transition temperature is referred to as the volume phase transition temperature (VPTT) where the network transitions from swollen to a collapsed state. One very recent illustration of using the thermoresponsive behavior of hydrogels for bio-applications has been by Kim et al., where an elastic creasing instability was induced upon pNIPAm-co-sodium acrylate hydrogels that were polymerized and surface attached to patterned substrates. When the temperature was lowered below the VPTT, thus allowing the gel to swell, the rigid
substrate underneath enabled swelling only in the direction normal to the substrate. Due to the presence of the topographical patterns on the substrate, a biaxial compressive strain was induced in the gel in those particular areas. This behavior was exploited for selective capture of protein-coated particles, protecting enzymes, and encapsulating cells. Besides temperature, the swelling behavior of hydrogels can also be affected by a variety of triggering events (as listed above), which can be utilized for biological applications such as drug delivery,\textsuperscript{35} biosensing,\textsuperscript{36-37} or tissue engineering.\textsuperscript{38}

1.3 Microgels

One way in which hydrogel materials can be classified is by their size. Macrogels are “bulk” gels with dimensions of 1 mm and larger, whereas microgels are stable colloids ranging from about 100 nm to a few microns in diameter.\textsuperscript{39} Microgels, like macrogels, can also respond to environmental triggers such as temperature,\textsuperscript{40} pH,\textsuperscript{41} light,\textsuperscript{42} or a magnetic field.\textsuperscript{43} The remainder of this chapter is devoted to the discussion of microgels, and how they can be used to make tunable and complex polymeric films.

1.3.1 Microgel Synthesis

Microgels can be synthesized by either emulsion polymerization, or by the more common approach of precipitation polymerization. Precipitation polymerization, which is the method used for synthesizing all microgels in this dissertation, is performed by taking advantage of the thermoresponsive behavior of pNIPAm (or pNIPMAm).\textsuperscript{40,44} Before initiation, all monomers and cross-linkers are dissolved in water, purged of oxygen with nitrogen gas, and heated to approximately 70 °C. Oxygen needs to be eliminated from the solution because it is a radical scavenger that will form peroxo radicals that can react with itself or another propagating radical to form inactive products, thus termination the polymerization.\textsuperscript{45} There is a two-fold purpose to heating the solution to a high temperature: to ensure the initiator (typically ammonium persulfate) will form
sulfate radicals, and the solution will be above the LCST of pNIPAm (or pNIPMAm). Once the initiator is added to the solution, an oligoradical polymer chain starts to grow until it reaches a critical chain length in which it is entropically unfavorable for it to remain as a solvated chain due to the structuring of water. At this critical chain length, the chain collapses into a precursor particle, which then continues to grow into a mature particle by aggregating with other precursor particles, capture of other oligoradicals, contributing to a more mature particle, or by additional monomer accumulation. The synthesis is referred to as precipitation polymerization because the polymer precipitates out of solution to form a nucleation site for the growing particle. This method can form very monodispersed populations containing various co-monomers and cross-linkers. In addition, their size can be controlled by increasing initiator and surfactant concentrations to generate small nanogels, or by temperature ramping to fabricate larger microgels up to 5 µm in diameter. Emulsion polymerization is best for microgels that contain relatively high amounts of hydrophilic monomer. Copolymerization of high percentages of hydrophilic comonomers can result in a growing oligoradical chain that does not effectively collapse into a nucleation site. Emulsion polymerization is also a better alternative to precipitation polymerization for synthesizing microgels that contain biological molecules that could be damaged at high temperatures.

1.3.2 Characterization

Microgels can be characterized using numerous methods. Because atomic force microscopy (AFM) is the main characterization tool exhibited in this dissertation, it will therefore be explained in greater detail in the next section. In addition, because dynamic light scattering is the most common used technique to interrogate the size of microgels when dispersed in solution, DLS will be discussed in greater detail in the section 1.3.2.2.
1.3.2.1 Atomic Force Microscopy

It must be noted that this discussion is not a general overview of AFM as a surface probe technique, but is only described in a manner that is relevant to the data shown in the following chapters so that the reader will have a better understanding of the work. In addition, different instrument manufacturers have different instrumental setups. The AFM utilized in this dissertation is an Asylum Research MFP-3D instrument. An extensive history and description of atomic force microscopy can be found elsewhere.\textsuperscript{48-50}

AFM is used to interrogate properties of surfaces, and offers many benefits over other types of microscopy. While optical microscopy requires little sample preparation and is also much simpler to use than AFM, light microscopy has a limited resolution of 0.2 µm due to the optical diffraction limit. Compared to other microscopy techniques that offer improved resolution over optical microscopy, such as scanning electron microscopy (SEM), scanning tunneling microscopy (STM), or transmission electron microscopy (TEM), AFM requires little sample preparation because the samples do not need to be conductive, coated with metal, or stained with a dye for improving imaging contrast. In atomic force microscopy, samples are studied using a probe that has a particular sharpness (the probes used in the following chapters have a radius of curvature of approximately 8 nm). The micro-machined probe is fixed orthogonally to the underside of a cantilever, which is suspended from a chip that is clamped to a holder. Optical lever detection is used to measure the forces that induce a deflection, or bending, of the flexible cantilever as it interrogates a surface (see Figure 1.1). In this manner, a super luminescent diode (SLD) or laser shines on the back of the cantilever, which is typically coated with a metal so that is reflective, and reflects onto a mirror which directs the beam to a multi-segmented photodiode. Very small changes in the cantilever deflection angle then change the position of the beam on the photodiode, which is in fact magnified because changes in cantilever angle, rather than a translational up and down movement, generate a larger relative reflective translation. The probe interacts with the
surface by scanning back and forth in the x-y direction, which is directed by piezos in the sample stage that enable small translational movements when an asymmetric current is applied to the piezo-electric material. A z-piezo is also in place in the “head”, which houses the cantilever hold, and monitors and controls the z-position of the cantilever.

Figure 1.1. AFM optical lever detection using a laser reflected from the back of a cantilever and directed to a photodiode detector. A cantilever is shown at two different deflection angles to illustrate the change that results on the detector.

There are two main types of AFM imaging modes: contact and non-contact mode. Contact mode means the tip is actually contacting the surface as it is scanned back and forth. As the cantilever deflects, a feedback loop applies a z-piezo voltage that changes the z-position to maintain a constant cantilever deflection. A topographical map is then generated based on the z-piezo voltage. This method is typically best for hard surfaces, and therefore not suitable for soft microgel films. In non-contact mode imaging, which is the imaging mode utilized in this dissertation, small forces between the probe and the sample, mainly van der Waals, are measured by the cantilever deflection. This mode is also described as “tapping” mode because the cantilever is driven to oscillate at or near its resonant frequency that is controlled by another piezo (the “shake-piezo”) present in
the cantilever holder. This oscillation also causes the deflection signal to oscillate. The feedback mechanism used here is “amplitude modulation” (AM), which means the z-piezo adjusts the cantilever as it is scanning the sample so that it maintains a constant amplitude of oscillation. These adjustments generate a trace of the sample, and are recorded as height data.

In liquid imaging, which is utilized extensively in Chapter 6, allows for interrogations of microgels when they are swollen in aqueous environments. Asylum Research has developed a technique known as “iDrive” that uses a special holder that is equipped with two electrically isolated and conductive spring clips that supply an AC voltage through a V-shaped two-legged cantilever. When a magnetic field is also applied to the cantilever, a Lorentzian Force actuates the cantilever in the direction orthogonal to both the current and magnetic field. Because the cantilever is directly actuated by the applied voltage and magnetic field, the in liquid imaging is much simpler because it allows the user to auto tune the cantilever while in liquid by eliminating the mechanical coupling between the shake-piezo and both the cantilever and fluid. The cantilevers used for in liquid imaging also have much lower spring constants (0.09 N/m) than the cantilevers used in ambient conditions (42 N/m), which is ideal for imaging soft water swollen microgels.

One must be aware of imaging complications such as contamination, tip breakage, or tip convolution during imaging using an AFM. While scanning a sample, the AFM probe can sometimes become contaminated by dust or dislodged sample material that can adsorb or adhere to the surface of the probe. It is apparent when this occurs because the image will show a repetitive artifact. Probes can also become dull or break when used for many scans or if crashed onto the surface, respectively. When there is contamination or tip breakage, the probe must be replaced. Tip convolution, which was a criticism of the work in Chapter 4, occurs when samples containing tall features, that are also very closely spaced, prevent the probe from reaching the bottom of the sample. The tips used
here are of a pyramidal shape that begins with the radius of curvature of approximately 8 nm and extends 10 – 15 μm upwards to underneath the cantilever. Therefore, in the case of Chapter 4, even though the distance between microgels was very small, their extremely short height (only 10 nm) allowed the cantilever to probe the underlying substrate because only taller particles would cause a tip convolution effect. To overcome this obstacle for samples that exhibit deep trenches, sharper AFM probes that have higher aspect ratios should be used.

1.3.2.2 Dynamic Light Scattering (DLS)

Light scatters when the path of a light beam deviates by interaction with a medium that contains “scattering centers” or non-uniformities. If the scattering center is smaller than the wavelength of scattered light, then the light scatters in all directions uniformly (Rayleigh scattering). Quasi elastic light scatter (QELS), otherwise known as DLS, uses this Rayleigh light scattering to determine the size of small objects that are diffusing through solution, such as colloidal hydrogels (microgels). In the solution sample that is exposed to a laser, various particles are diffusing due to Brownian motion, and as a result there are many beams of scattered light that interfere with one another. The scattered light that reaches the detector is affected by the incidence of constructive and destructive interference, and these fluctuations lead to an intensity versus time relationship. The light source must be monochromatic and coherent in order to observe this relationship. The scattering intensity time dependence is directly correlated to the microgel’s diffusion, which can be related to the size, or hydrodynamic radius ($R_h$), of microgels. Therefore, in principle, the fluctuation in scattered light intensity will increase as the size of the particle decreases because smaller particles diffuse more quickly through solution. An autocorrelation function is used to plot the average change in light intensity over time, plotted as a function of delay time or specific time interval ($\tau$), and this information illustrates how quickly the light intensity changes with time. The
autocorrelation function plot should be a single exponential decay for a mono-dispersed particle sample, expressed as:

\[ g(\tau) = e^{(-\tau/\kappa)} \]

From this decay, the diffusion coefficient (D) can be calculated using the time constant (κ) that is associated with the autocorrelation function:

\[ \kappa = 1/(2Dq^2) \]

where D = the translational diffusion coefficient and q = the wave vector of the diffusing particle. The diffusion constant, together with the temperature (T), solvent viscosity (η), and Boltzmann’s constant (k_B), is used to determine the microgel R_h using the Stokes-Einstein equation:

\[ D = \frac{k_B T}{6\pi \eta R_h} \]

Therefore, the DLS is used to measure a spherical particle or molecule’s hydrodynamic radius by determining it’s diffusion in solvent.

1.3.3  Microgels As Thin Film Building Blocks

As mentioned previously, hydrogels are very versatile and useful materials, especially when interfacing with biology. This is due to their capacity to be tuned in terms of their chemical, mechanical, and responsive properties. These properties can also be incorporated with microgels. Additionally, microgels typically show faster deswelling kinetics than macrogels of similar composition, enabling microgel films to respond more rapidly to external stimuli. The slow deswelling of macrogels is due to shrinkage of the outer layer that forms a “skin” and inhibits water transport outwards from the interior, which can be minimized by having a smaller colloidal structure. For film assembly, microgels can be thought of as individual building blocks in a bottom-up approach. By utilizing this bottom-up approach, both chemically and morphologically complex interfaces can be generated, which can be difficult to accomplish using a macrogel. For bulk gels, the ability to fabricate such complexity is typically limited to microfabrication.
patterning technology, which has a resolution on the range of 2 – 10 µm, and requires techniques such as photolithography that can be tedious and impractical.\textsuperscript{51}

A bottom up assembly approach can offer more chemical complexity to an interface. Various different chemical functionalities can be incorporated with hydrogel materials,\textsuperscript{21} and therefore can also be applied to microgels. Orthogonal chemistries can even be incorporated within a single microgel.\textsuperscript{52-53} Spatial control of functionalities within a single microgel can also be accomplished using a core-shell construct,\textsuperscript{54} which localizes particular chemistries either in the core or the shell of the particle. Furthermore, assortments of various microgels containing these different chemistries may be assembled simultaneously on surfaces in a mosaic fashion,\textsuperscript{55} thus facilitating the formation of polymeric films that contain a multitude of properties (Figure 1.2).

![Figure 1.2. Mosaic assembly using a variety of different microgels for a complex film.](image)

Microgel thin films can also offer interfaces with controlled topography. One such example has been demonstrated by Dawson and coauthors.\textsuperscript{56} In this work, microgels are phase separated into discrete areas of particle rich and particle poor regions, dictated by a depleting agent to induce attractions between particles upon solvent evaporation, which made surfaces that contained micron-sized pores. These micron-scale topographical features are simply controlled by changing the size of the microgels. Another example of using microgels to make interfaces with a topographically rich construct is illustrated by Serpe et al.\textsuperscript{31-33} In this work, anionic microgels were assembled using a polyelectrolyte layer-by-layer (LbL) approach, as discussed in section 1.1, with a
linear polycation acting as the “glue” layer between microgels. The polyvalent interactions and polymer interpenetrations play an important role in the ability for these three-dimensional materials to form continuous films that are sufficient for blocking macrophage adhesion and modulating the foreign body response to medical devices (Chapters 3 & Appendix A), and enable microgel multi-layered films to self-heal when assembled on soft supports that are then subject to significant damaging events (Chapter 5).

Due to the characteristics described above, and from what will be described in the following chapters, it is apparent that microgels are an enabling polymeric thin film building block. Compared to other particle systems, including rigid spheres (polystyrene microspheres, silica particles, metallic particles, or quantum dots), and other soft particle systems (liposomes, or block copolymer micelles), microgels offer a higher range of properties that have facilitated their use in a variety of useful and interesting applications including colloidal crystals,\textsuperscript{57-59} tunable microlenses,\textsuperscript{60-61} drug delivery,\textsuperscript{62-63} and nonfouling films.\textsuperscript{64-67}

1.4 References


CHAPTER 2
HOST RESPONSE TO IMPLANTED MATERIALS

2.1 Introduction

Studies centered on the interface between biological environments and synthetic materials have been of great interest for many years, and have been of immense importance for improving the performance and lifetime of medical implants. Implanted materials can serve as prostheses, drug-delivery agents, or for sensing important molecules or signals throughout the body. Whereas medical implants have come quite a long way over time due to significant advancements, unfortunately there continues to be little understanding as to what precisely occurs at the biological and material interface. Most implants suffer from dysfunction either over short time periods, due to protein adsorption or cellular adhesion that can impede sensing or drug delivering capabilities, or over long periods of time, due to chronic inflammation and scar tissue formation. These problems have yet to be effectively solved.

It is generally known that when a foreign object is implanted into a mammalian body, the surrounding biological environment recognizes the material as foreign and undergoes a “foreign body response” (see Figure 2.1). This response first involves non-specific protein adsorption, or fouling, to the surface of the biomaterial within a few minutes to an hour. The adsorbed proteins provide a scaffold upon which white blood cells, or leukocytes, which are present in high numbers during the wound healing process, can attach by cell membrane receptors known as integrins. Integrins recognize specific proteins that adsorb to surfaces, such as fibronectin, fibrinogen, or Immunoglobulin G (IgG), and this interaction is also highly dependent on the conformation of the adsorbed protein due to the accessibility of specific amino acid sequences that integrins specifically bind. When surface receptor integrins bind to a surface, they cluster
together to form focal adhesions,\textsuperscript{8} which trigger intracellular signaling that enables cells to adhere and spread on surfaces through methanotransduction.\textsuperscript{9-11} There are two main types of leukocytes that infiltrate the inflammatory site during the foreign body response: neutrophils and monocytes (macrophages). Neutrophils are the first to arrive at the site of inflammation, and their function is to phagocytose microorganisms and foreign materials.\textsuperscript{12} Neutrophils are typically cleared from the site within a few days due to a loss of neutrophilic chemotactic signals that are only present early on in the wound healing process.\textsuperscript{1} Monocytes also adhere to the surface of foreign objects at early time points, but then quickly differentiate into macrophages that persist at the inflamed site for several months.\textsuperscript{13} An analogous biological response, which also involves the adsorption of proteins followed by cellular adhesion, is the mononuclear phagocytic system (MPS), which applies to blood circulating nanoparticles that are greater than 10 nm in diameter.\textsuperscript{14} Proteins adsorb to the surface of the nanoparticles during a process known as opsonization, which makes them visible to macrophage cells.\textsuperscript{15} These cells then interact with the opsonized proteins and engulf the material by phagocytosis, which results in a break down of the engulfed material. However, with a large object such as an implant, which is much larger than the macrophage itself, phagocytosis is impossible. Therefore, cells adhere and spread on the surface of the implant over the span of a few days, and after about a week has passed, the cells fuse together into a foreign body giant cell.\textsuperscript{16} These cells also release small molecules known as cytokines that facilitate the wound healing response, and activate and attract more leukocytes to the site of inflammation.\textsuperscript{17} Over several weeks, a fibrous collagen capsule forms around the biomaterial that segregates the material from the rest of the body.\textsuperscript{2} This foreign body response severely affects the function of many implants such as biosensors,\textsuperscript{18} neural probes,\textsuperscript{19} and orthopedic implants.\textsuperscript{20}
Figure 2.1. Foreign body response to implanted materials. Note, images in figure are not drawn to scale.

2.2 Non-Fouling Materials

Attempts at controlling inflammation and promoting wound healing have been aimed at taking what knowledge we have about the biological response to a foreign material (discussed in section 2.1) and using that knowledge to modify the surface of biomaterials. One of these approaches has been geared towards controlling the initial step of the host’s response to foreign materials, which is non-specific protein adsorption. These surfaces are termed “non-fouling”. A reduction in protein adsorption should consequently also result in a decrease in the number of leukocytes that adhere to the surface, followed by a reduced fibrous capsule. Some surface modifications come from existing biological components, such as phospholipids and saccharides, and have been shown to resist fouling. However, to better understand what material traits actually
contribute to a non-fouling surface, George Whitesides and coauthors surveyed chemically modified surfaces for their potential at reducing protein adsorption. Using self assembled monolayers of various terminally-modified alkanes, four main characteristics were found to maximize anti-fouling behavior: (i) polar functional groups, (ii) hydrogen bond accepting groups, (iii) a lack of hydrogen bond donating groups, and (iv) charge neutrality. In light of these observations, it is no mystery as to why one of the most widely accepted molecules used for the control of protein adsorption is poly(ethylene glycol) (PEG), alternatively known as poly(ethylene oxide) (PEO), which possesses these four characteristics shown to resistant protein adsorption. In addition, work by Jeon et al. suggest that the anti-fouling behavior of PEG can be attributed to it’s hydrophilicity, which allows PEG to obtain a high level of flexibility and extended chain conformation when in aqueous solutions. Therefore, when proteins interact with the surface by van der Waals or hydrophobic interactions, the extended PEG chains can compress and generate a repulsive force which can counteract the attractive force with the protein. Consequently, the ability of PEG to “repel” proteins is highly dependent on chain molecular weight, surface chain density, and chain conformation of the PEG. A higher polymer chain molecular weight has a higher chain flexibility, which contributes to a better non-fouling interface. PEG chain density and chain conformation are highly dependent on one another, and their effect on protein adsorption is inversely related (Figure 2.2). While a low density allows for greater movement of PEG chains, which take on a “mushroom” configuration (a), the low coverage leads to exposed areas where proteins can easily adsorb. A high density of PEG chains results in better coverage of the surface (b), but also contributes to a restricted chain conformation that leads to reduced steric hindrance properties of the film. Therefore, there is an ideal balance between PEG chain conformation and coverage that is optimal for maximum resistance of protein adsorption. Ma et al. illustrated an interesting approach to this balance using a “bottle-brush” polymeric construct consisting of a methacrylate backbone containing
oligo(ethylene glycol) side-chains (OEGMA).\textsuperscript{31} By growing OEGMA from a surface using surface-initiated atom transfer radical polymerization (SI-ATRP) from an ATRP initiator-functionalized alkane thiol, they found that in a mixed SAM film a relatively low molar ratio of initiator-containing alkane thiol to methyl-terminated thiol (0.2) was needed to significantly reduce fibronectin adsorption (down to below the surface plasmon resonance detection limit). The ability for these low brush density constructs to resist protein adsorption was attributed to the extension of orthogonal oligo(ethylene glycol) side chains from the brush backbone.

![Figure 2.2](image)

**Figure 2.2.** PEG brushes (black) in low density (a) and high density (b), and their ability to resistant adhesion of various proteins (colored).

PEGylation was first introduced in the modification of proteins and other therapeutics to increase their blood circulation times, thus improving their bioavailability, for drug delivery.\textsuperscript{32-34} PEGylation was also introduced in nanoparticle drug delivery systems for the same motivation. Studies have also been conducted on the use of PEG for controlling non-specific protein adsorption to macroscopic surfaces, such as self-assembled monolayers of oligo(ethylene oxide),\textsuperscript{24-26} or the SI-ATRP brush approached mentioned earlier,\textsuperscript{31} which have all been shown to dramatically decrease protein adsorption to surfaces. Additionally, there have been several attempts to use PEG-containing non-fouling coatings, which have shown reduced protein adsorption to surfaces \textit{in vitro}, to further control biological interactions with a macroscopic surface \textit{in vivo}.\textsuperscript{35-39} However, many of these studies have shown to be unsuccessful at controlling
the foreign body response in \textit{vivo}. For example, there has been extensive work performed by Horbett and coauthors in using radio frequency plasma deposition of tetrathylene glycol dimethyl ether (tetraglyme) to modify implantable surfaces.\textsuperscript{40-41} These modified surfaces showed significant reduction in the adsorption of fibrinogen, albumin, and IgG, with concentrations found to be as low as 2 ng/cm\textsuperscript{2} \textit{in vitro}. However, when this promising coating material was plasma deposited on a fluorinated ethylene propylene copolymer (FEP) surface and implanted into mice, a fibrous capsule had formed around all the implants after four weeks.\textsuperscript{35} The capsule thickness was also no different than uncoated FEP. Therefore, a simple reduction in protein adsorption does not necessarily result in a reduced foreign body response \textit{in vivo}. In fact, the inability to directly draw conclusions \textit{in vitro} that will translate to \textit{in vivo} studies is one of the most challenging disconnects that the biomaterials community continues to face today.

Because hydrogels have been shown to possess characteristics that make them favorable for interfacing with biology (as discussed in \textbf{Chapter 1}), research has also been involved in incorporating PEG into hydrogel films to serve as anti-fouling biomaterial interfaces.\textsuperscript{42-44} PEG hydrogels have been used to prevent surgical adhesions at a wound site,\textsuperscript{45} to prevent protein adsorption and cellular adhesion to silicone rubber,\textsuperscript{46} and even as a network to house quantum dots for sensing in biological environments.\textsuperscript{47} In the next section, PEG is used as a co-monomer in the synthesis of microgels, and the non-fouling behavior of these microgels is discussed.

\section*{2.3 PEG-Modified Microgels}

Due to the widespread acceptance and promising results that the incorporation of PEG has offered for a non-fouling material, Gan et al., Nolan et al., and Singh et al. have all copolymerized PEG into microgels. Dr. Gan incorporated PEG chains by the copolymerization of pNIPAm and PEG acrylate (MW=1000). These microgels showed resistance to bovine serum albumin (BSA) adsorption, with the reduction in protein...
adsorption being enhanced by increasing the number of PEG grafts.\textsuperscript{48} Compared to a non-PEGylated microgel, particles containing 40 wt\% PEG showed more than a four-fold decrease in BSA adsorption. Furthermore, non-PEGylated microgels in the deswollen state exhibit twice the level of protein adsorption compared to their swollen state. This effect was attributed to the hydrophobic adsorption of proteins onto microgels, as the microgels are hydrophobic above the VPTT (explained in Chapter 1). However, collapsed microgels containing PEG were still able to decrease BSA adsorption. Specifically, microgels with only 10 wt\% PEG contributed to a more than two-fold decrease in protein adsorption at 42 °C compared to non-PEGylated microgels. It is also important to point out that the incorporation of PEG increases the microgel VPTT, shifting from 31 °C for BIS cross-linked pNIPAm microgels to approximately 36 °C for microgels containing 40 wt\% PEG, and also broadens the phase transition. This phenomenon is due to the increased hydrophilic composition of microgels by PEG copolymerization, thus requiring more thermal energy to undergo phase separation from the aqueous environment.

Nolan et al. later demonstrated a similar protein adsorption reduction (over four-fold compared to non-PEGylated microgels) when only 20 wt\% PEG diacrylate cross-links (MW=700) were co-polymerized within the microgel.\textsuperscript{49} This effect was observed at both room temperature and 37 °C. Because many cells show recognition of and adherence to surface-adsorbed proteins, it was hypothesized that a reduction in protein adsorption would also reduce cellular adhesion. Consequently, \textit{in vitro} experiments were conducted to illustrate this concept. When PEG diacrylate cross-linked microgels were copolymerized with acrylic acid and spun-cast onto an amine-functionalized glass substrate, the amount of fibroblast adhesion was dramatically reduced when compared with tissue culture polystyrene.

Due to these promising results, efforts were then focused on the regulation of macrophage adhesion. Macrophages are actively involved in inflammation, wound
healing, and the foreign body response to an implanted material. Poly(ethylene terephthalate), or PET, was chosen as a relevant model biomaterial to which non-fouling microgels would be attached. PET is also a suitable substrate for in vivo implantation, being commonly used in various biomedical applications such as sutures and sewing cuffs for heart valves, and is known to elicit both acute and chronic inflammatory responses. Neetu Singh used a tailored surface modification chemistry to covalently attach non-fouling PEG diacrylate cross-linked microgels to a PET substrate. The microgel film provided complete uniform coverage on the surface, thus effectively blocking the underlying substrate from the environment, and was highly conformal to imperfections that are present throughout the PET. In collaboration with the Andrés García group at Georgia Tech, cell studies were performed on the microgel coated PETs by Amanda Bridges. Both bare PETs and microgel-coated PETs were incubated in cell culture media containing serum and macrophages. Whereas the bare PET elicited high levels of cellular adhesion and spreading after 48 hours, the microgel-coated PET displayed no macrophage adhesion during this time period. The cellular resistant behavior of the microgel-modified PET was attributed to the lack of protein adsorption on the surface, which is necessary for cellular adhesion.

To evaluate non-fouling microgel coatings in vivo, Amanda Bridges also conducted studies on implanted microgel-modified PETs in mice. After implantation in the murine intraperitoneal space for 48 hours, there was an observed reduction in leukocyte adhesion and a decreased expression of pro-inflammatory cytokines to microgel-coated PET when compared to bare PET. These studies illustrate the effectiveness of PEG-modified microgels at minimizing the host’s acute inflammatory response to foreign materials, which once again is attributed to the non-fouling, or protein resistant, properties of these microgels. Therefore, it is apparent that microgel-coatings are able to control biological responses both in vitro and in vivo. In addition, chronic inflammatory responses to microgel-coated PETs were evaluated by Amanda Bridges and
Rachel Whitmire of the Andrés García group. After four weeks of subcutaneous implantation in the rat dorsum, the capsule thickness, cell density, and relative numbers of macrophages at the material-tissue interface were assessed. Microgel-coated PETs resulted in the formation of a thinner fibrous capsule and decreased density of capsule-associated cells, by 22% and 40% respectively, compared to unmodified PETs. These observations suggest that microgel coatings contribute to reduced chronic inflammation, which was determined by the formation of a thinner and less dense capsule. Attempts at using other types of coatings, such as dihydroxypropyl methacrylate, PEG, and phosphorylcholine-based polymers, have also shown a reduction in capsule thickness that is comparable to non-fouling microgel coatings. However, the microgel-coated PET showed a similar total number of macrophages to that of bare PET, and the relative number of macrophages compared to cells within the capsule was almost twice for microgel-coated PETs than bare PETs. Additional experiments need to be conducted, such as the characterization of macrophage phenotype, to see if perhaps a different type of macrophage is present at the material surface. Overall these results illustrate the complex relationship between acute and chronic processes of the foreign body response, which to this day is still poorly understood. In addition, these could aid in a better understanding of the host response to foreign materials.

2.4 Outlook for Non-Fouling Microgels

Non-fouling microgels are promising colloidal materials that have the potential to provide control over a host’s biological response to biomaterials. These non-fouling coatings can be assembled at an interface and prevent non-specific protein adsorption, which consequently leads to reduced cellular adhesion and a thinner fibrous capsule around the non-fouling microgel-coated material. The research discussed in this chapter is only a recount of the work performed by others that serves as background for understanding other chapters in this dissertation. As will be seen in Chapters 3 and 5.
and in Appendix A of this dissertation, non-fouling microgels inspired a large portion of this body of work. More specifically, they led to non-fouling multi-layered films (Chapter 3), coatings on medically implantable devices (Appendix A), and self-healing films (Chapter 5).

2.5 References


CHAPTER 3

MULTI-LAYERED NON-FOULING MICROGEL FILMS

3.1 Introduction

Poly(ethylene glycol)-containing microgels, or non-fouling microgels, were discussed in Chapter 2, and have been shown to successfully reduce both acute and chronic responses that transpire once a foreign object is implanted into a mammalian host.\textsuperscript{1-2} Though non-fouling microgel coatings show great promise towards control over the foreign body response, and also resulted in a thinner fibrous capsule after four weeks of implantation compared to an uncoated biomaterial, the coated substrate was still able to elicit an unfavorable response from the host. A relatively high number of white blood cells, or macrophages, were present in the fibrous capsule encapsulating the microgel-coated biomaterial, when compared to the uncoated biomaterial.\textsuperscript{2} These non-fouling microgel coatings could benefit from additional modifications that could lead to more control over the response of these macrophages. Therefore, an ultimate goal for non-fouling microgel films is to generate a background “stealth” surface that prevents non-specific protein adsorption, while also exhibiting more control over specific cellular adhesion and other consequences of the foreign body response (Scheme 3.1). Microgels have the capability to be modified and tuned for different applications (see Chapter 1), and therefore it is possible to strive for such a complex interface using these materials.
Scheme 3.1. Illustration of complex interface where non-specific protein adhesion is reduced and cellular adhesion peptide-containing microgels direct cellular adhesion.

Hydrogel films have previously been used to gain control over cellular adhesion in several ways. An early example of this concept was demonstrated by Okano et al. where the thermostresponsivity of a pNIPAm hydrogel film was utilized to create a reversible cell substrate.\(^3\) As discussed in Chapter 1, pNIPAm exhibits a volume phase transition at approximately 31 °C in water whereby upon an increase in temperature from below to above this temperature the polymer transitions from a swollen to collapsed state. At a physiological temperature of 37 °C, cells adhere to the hydrophobic and collapsed film. When the incubation temperature is dropped below the lower critical solution temperature of the film, the cells experience a hydrophilic or swollen surface and consequently detach. The original theory behind this cellular adhesion control was centered around the general phenomena that cells do not adhere well to highly hydrophilic surfaces.\(^4\)\(^5\) However, more recent studies suggest that cellular attachment is more dictated by a relationship between the stiffness of the pNIPAm network and cell cytoskeletal reorganization.\(^6\) By employing tyrosine kinase inhibitors, actin filament stabilizers, or actin filament depolymerizers, which render cells unable to reorganize their cytoskeleton or carry out intracellular signal transduction, the cells were unable to respond to the change in the surface softness when the temperature was lowered, and
consequently no cell detachment was observed. These results suggest that cell
detachment is mediated by intracellular signaling and cytoskeletal restructuring, and is in
agreement with studies that illustrate increased cellular adhesion on stiffer substrates due
to cytoskeletal reorganization.\textsuperscript{7} In addition to using mechanical changes of the substrate
to control cellular adhesion, biologically relevant molecules, such as cellular adhesion
moieties,\textsuperscript{8-11} growth factors,\textsuperscript{12-13} and extracellular matrix (ECM) molecules\textsuperscript{12,14-16} can be
attached to a hydrogel network. One well-known peptide sequence that promotes cellular
adhesion is RGD (arginine, glycine, aspartic acid).\textsuperscript{17} RGD is a sequence found in many
extracellular matrix proteins. Cells adhere to RGD by integrin receptors, thus allowing
them to attach to a surface and spread. Various ECM matrix proteins have been
covalently attached to a hydrogel to serve as a network for which cells can adhere, such
as to repair broken bone,\textsuperscript{18} or modulate marrow stromal cell (MSC) attachment.\textsuperscript{8} Cellular
adhesion and spreading can also be controlled by altering the topography of the film,
which has been demonstrated by Dawson and coauthors on a microgel assembly (refer to
section 1.3.3 for a description on how these films were assembled).\textsuperscript{19} By utilizing this
phase separating technique to generate interfaces of varying topology on the micron-
scale, Dawson and coauthors were able to observe the different cell morphologies that
were directed by these changes.

In the work discussed here, studies were aimed at enabling controlled cellular
adhesion using non-fouling microgels that would contain cellular adhesion peptides such
as RGD. Unfortunately, the coating process discussed in Section 2.2 that was introduced
for the model biomaterial (PET)\textsuperscript{20} had several disadvantages that presented hurdles for
providing a cell adhesive surface containing peptides:

1. Microgel deposition was performed under non-physiological conditions, which is
not conducive for the incorporation of peptides.
2. The use of plasma etching to modify the surface can make the surface of some substrates brittle, therefore compromising the integrity of the original implant.

3. The films were deposited using spin-coating, which is a tedious and wasteful process that is impractical for high throughput of samples made from materials that contain expensive peptides.

The work here discusses the significant modifications that were necessary for assembling films of peptide-containing non-fouling microgels. Also discussed in this chapter are the major conclusions from these film fabrication methods, and an assessment of the film’s non-fouling behavior.

3.2 Experimental

3.2.1 Materials

All reagents were purchased from Sigma-Aldrich unless otherwise specified. The monomer N-isopropylacrylamide (NIPAm) was recrystallized from hexanes (J.T. Baker) and dried under vacuum prior to use. The cross-linker poly(ethylene glycol) diacrylate with average Mw = 575 (PEGDA575), co-monomer acrylic acid (AAc), surfactant sodium dodecyl sulfate (SDS) and initiator ammonium persulfate (APS) were used as received. Buffer chemicals sodium dihydrogen phosphate monohydrate (Fisher Scientific), 2-(N-morpholino)ethanesulfonic acid (MES), sodium chloride (Mallinckrodt), and sodium hydroxide were used as received. The surface modification reagent 3-aminopropyltrimethoxysilane (APTMS, TCI America) was used as received. Carbodiimide coupling chemicals N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), and hydroxylamine hydrochloride were used as received. Poly(diallyldimethylammonium chloride) high molecular weight 400,000-500,000 (PDADMAC) was used as received. Glass disks of
12-mm diameter were purchased from Bellco Glass. Absolute (200 proof) ethanol was used as received from EMD Chemicals Inc. All water used throughout this investigation was house distilled, deionized to a resistance of at least 18 MΩ (Barnstead Thermolyne E-Pure system). IC-21 murine macrophage cell line was obtained from ATCC (Manassasa, VA) and cultured as directed. RPMI-1640 media was purchased from Invitrogen Corporation (Carlsbad, CA) and/or ATCC (Manassas, VA), supplemented with 10% Fetal Bovine Serum (Invitrogen) and 1% penicillin streptomycin (Invitrogen) and used to culture the IC-21 cell line. 100 mm and 150 mm tissue culture polystyrene dishes (Corning Inc., Corning, NY) were used to culture cells. PBS (with and without Calcium and Magnesium) was obtained from Invitrogen. Versene (Invitrogen) was used to dissociate the cells from the dishes. 12-well plates from Corning (Corning, NY) were bought via Sigma-Aldrich (St. Louis, MO) and used for the cell culture experiments. Calcein and Ethidium homodimer were bought from Invitrogen Corporation (Carlsbad, CA) and used at final concentrations of 4 µM to stain for live and dead cells. Peptides were purchased from AAPPTEC (http://www.aapptec.com/home.aspx) and stored in a subzero freezer. Biotin hydrazide was used as received. Streptavidin coated polymer beads (0.97 µm diameter) were purchased from Bangs Laboratories, Inc. and kept refrigerated before use.

3.2.2 Microgel Synthesis

Non-fouling microgels were synthesized using aqueous free radical precipitation polymerization. The synthesis solution contained a total monomer concentration of 121 mM with molar compositions of 73% NIPAm, 3% PEGDA575, and 24% AAc was used. Surfactant SDS was used in concentrations of 0.17 mM. NIPAm and SDS were dissolved in 49 mL of deionized water and filtered through Whatman #2 filter paper in a vacuum filtration system. The aqueous solution was then transferred to a three-neck round bottom flask and purged with N₂ for approximately 1 hour while the solution was heated to 70
°C. Ten minutes before initiation, the PEGDA575 and AAc were added to the warm solution via transfer pipette. The initiator APS (0.0114 g), used in a total final concentration of 1 mM, was dissolved in 1 mL of deionized water and added to initiate the polymerization. The reaction was allowed to proceed for 4 hours at 70 °C under a blanket of N₂. After synthesis, the microgel solution was filtered again through Whatman #2 filter paper and then purified by centrifugation with five times removal of the supernatant and redispersion of the ensuing pellet in water.

Core-shell microgels were synthesized by first synthesizing a core microgel containing 100 mM total monomer concentration with molar compositions of 98% NIPAm and 2 mol% PEGDA575. Synthesis conditions and surfactant and initiator concentrations were same as for the synthesis described above. A shell was polymerized around the core using a method that utilizes the core as a nucleation site for polymerization of additional polymer, which results in a shell. The total monomer concentration of the shell was 40 mM with molar compositions of 70% NIPAm, 3.5% PEGDA575, and 26.5% AAc. A surfactant of 1 mM was also used. To 20 mL of unpurified core synthesis, the shell solution was added with enough water to make a 100 mL final solution volume. The shell synthesis was initiated with 1 mM APS and allowed to proceed in the same manner as described above for four hours at 70 °C. Core-shell microgels were purified using centrifugation.

3.2.3 Substrate Functionalization and Microgel Film Assembly

12-mm diameter glass coverslip disks were placed in a ceramic glass slide holder and cleaned using a sequential solvent sonication method. A sequential solvent sonication method proceeded with the following solvent sequence using a Branson 2510 Ultrasonicator (42 kHz +/- 6% output): 30 minutes in dilute soapy (Alconox) water, 15 minutes in deionized water, 15 minutes in acetone, 15 minutes in absolute ethanol, and 15 minutes in isopropyl alcohol. Afterwards, the glass was immediately equilibrated for 30
minutes in absolute ethanol and 1% by volume APTMS was added. The glass was incubated with the APTMS-ethanol solution for 2 hours under gentle agitation. The glass was then rinsed with a 70% aqueous ethanol solution and deionized water, and then dried under a gentle stream of N₂.

Cleaned and dried glass disks were individually placed at the bottom of 24-well plates and high ionic strength PBS was immediately added. The glass was allowed to equilibrate for 30 minutes, and the buffer was then replaced with a 0.1 mg/mL solution of microgels in pH 7.4 phosphate buffer containing 100 mM ionic strength (PBS). The well plates were placed immediately across a counter-weighted well plate in an Eppendorf 5804R centrifuge equipped with a plate-holding rotor. Films were centrifugally deposited at a maximum rotor speed of 2250 × g for five minutes. After deposition, the films were rinsed with deionized water and dried under a gentle stream of N₂. Afterwards, the monolayers were covalently attached to the amine-functionalized glass by activating the acids on the particles. To accomplish this, EDC/NHS bioconjugation chemistry was employed.²² A solution containing 2 mM EDC and 5 mM NHS was prepared in 100 mM MES buffer (pH 5) and allowed to react with the non-fouling microgel film for 2 hours at room temperature. After rinsing the films with water, they were exposed to a 10 mM solution of hydroxylamine in MES buffer for 10 minutes to quench the EDC/NHS reaction. The films were then rinsed with water to remove excess reagents.

In the past, the Lyon group has amply demonstrated the use of microgels in the fabrication of multi-layered thin films.²³⁻²⁶ In this study, microgel monolayer films were constructed using active deposition in the same fashion as described above. To add an additional layer, a 0.1 monoM (molar concentration of monomer) solution of PDADMAC (MW = 400,000 – 500,000) was added to the well and allowed to adsorb to the microgel film for 30 minutes. The films were then washed five times with deionized water. Another layer of microgels was then added to the well and centrifuged onto the surface, as described above. This process was repeated until four microgel layers were
deposited. For peptide-containing films, an addition layer (layer 5) of peptide-conjugated core-shell microgels was centrifugally deposited after passive adsorption of a fourth layer of PDADMAC.

3.2.4 Peptide Conjugation

Non-fouling microgels were conjugated with either RGD or RGE peptide sequences via carbodiimide coupling chemistry between the acid-containing microgels and primary amine on the N-terminus of the peptide. Microgels were first redispersed in 100 mM pH 5 MES buffer by centrifugation, and then a ten-fold excess of EDC and NHS, also dissolved in 100mM MES buffer pH 5, were added to the microgel dispersion and allowed to activate the acids for 30 min. The microgels were then centrifuged twice to remove unreacted EDC and NHS, followed by addition of peptide that amounted to a 1 mol % of total available acids in the same MES buffer. The conjugation was allowed to proceed for 2 hours at room temperature on a shaker table. Afterwards, unconjugated peptide was removed by three cycles of centrifugation, and a 10 mM solution of hydroxylamine in the same MES buffer was added for 10 min to quench any remaining activated acids. Microgels were purified and redispersed in high ionic strength PBS, by centrifugation five times, in preparation for film deposition. Core-shell microgels were conjugated in the same manner, except the scrambled cellular adhesion peptide was RDG instead of RGE, and both 1 mol% and 10 mol% of total available acids were conjugated with peptide.

3.2.5 Ligand Bioavailability Experiments

Non-fouling microgels and core-shell non-fouling microgels were conjugated with biotin using carbodiimide coupling chemistry as described in Section 3.2.4. More specifically, 10 µL of a 5x10^{-4} mg/mL solution of biotin hydrazide in DMSO was added to 1 mg/mL solutions of microgels that were dispersed in MES buffer with a ten-fold
excess of EDC and NHS. After microgels were purified via centrifugation and redispersed in PBS containing 100 mM ionic strength, microgels were centrifuged onto amine-functionalized glass (as described in Section 3.2.3). To test the accessibility of biotin in the biotin-conjugated microgels, the streptavidin coated bead stock solution was diluted 100-fold in PBS of 100 mM ionic strength and incubated with the microgel films for 24 hours either in at 4 °C or 37 °C. Afterwards, films were rinsed with deionized water, dried with N₂ and imaged using brightfield optical microscopy using the 100x objective. Bright field optical microscopy was performed using an inverted microscope equipped with a Cooke Corporation PixelFly black and white CCD camera.

3.2.6 Atomic Force Microscopy

Microgel films were imaged using an Asylum Research MFP-3D Instrument (Santa Barbara, CA). Imaging was performed and processed using the MFP-3D software under the IgorPro (WaveMetrics Inc., Lake Oswego, OR) environment. Non-contact mode aluminum-coated silicon nitride cantilevers were purchased from NanoWorld (force constant = 42 N/m, resonance frequency = 320 kHz). All images were taken in air under ambient conditions.

3.2.7 In Vitro Cellular Adhesion Studies

The in vitro studies were performed by Rachel Whitmire from the Andrés Garcia group at Georgia Tech. Microgel multi-layer films were sterilized after assembly in 70% ethanol aqueous solution. Before use with cells, films were washed three times in sterile PBS, and then equilibrated in fresh PBS for at least 1 hour before use. A murine peritoneal macrophage cell line, IC-21, was employed to test for adhesion to the multi-layer films in vitro. Macrophages are one of the primary mediators of the inflammatory response, and can fuse to form the foreign body giant cells that make up a significant portion of the fibrous capsule around an implanted biomaterial. The use of a macrophage
cell line is standard for examining non-fouling behavior in vitro due to this cell type's adhesive nature and role in the body's response to a biomaterial. The IC-21 cell line is an established murine peritoneal macrophage line that expresses many of the standard macrophage surface proteins and maintains the phagocytic and cytolytic behaviors characteristic of untransformed macrophages. IC-21 cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (PS). The cultures were grown at 37 ºC with 5% CO2. The cells were removed using Versene, counted by hemacytometer, then diluted to a concentration of 200,000 cells/1.5 mL media. The microgel films were placed in a sterile 12-well culture dish, and 1.5 mL of cells/media were added. The films were incubated at 37 ºC for 4 hours, after which the excess media/cells were aspirated and samples were transferred to new wells and fresh media. Samples were incubated overnight and stained the next day with 4 µM calcein and ethidium homodimer in PBS. Samples were imaged at 20x or 10x magnification with a Nikon Eclipse E400 upright microscope (Nikon Instruments, Inc., Melville, NY). Images were taken with Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI). Eight representative images were taken per sample, with three samples per group. Cells were counted using the public domain NIH ImageJ program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The cell count was averaged over all representative images of the same sample type, and the error bars shown represent the standard error for the group of three samples.

3.3 Results & Discussion

Non-fouling PEG-containing microgels have been shown to dramatically reduce both protein and cellular adhesion to surfaces, and also reduce the thickness of the associated fibrous capsule in vivo.1-2 From these past studies, we have learned a great amount about non-fouling microgel coated biomaterials, and are looking to generate interfaces that can direct specific cellular adhesion for better integration of biomaterials.
with the biological environment. In order to accomplish this task, we looked to incorporate cellular adhesion peptides with non-fouling microgels. However, the current method for film assembly and covalent attachment to poly(ethylene terephthalate) (PET) was in need of several alterations. PET was functionalized by grafting acrylic acid brushes from the surface after argon plasma exposure, and aminobenzophenone was conjugated to these acids to provide a photo-affinity label for covalent attachment of microgels. The non-fouling microgels, which also contained acrylic acid side chains, were then spin-coated onto the functionalized PET surface. The microgels needed to be dispersed in a low pH buffer (below the pKa of the acids, which is approximately a pH of 4) so that they would not be repelled from the surface of the AAc-functionalized PET. After exposure of UV light, the microgel film was permanently attached to the surface of the PET. However, in order to provide a favorable environment for peptide-conjugated microgels, a buffer of approximately pH 7.4 must be used as the microgel dispersion medium. When these microgels are dispersed in such a physiologically-relevant buffer, which is above the pKa of acids both on the microgels and the polymer brushes on PET, the microgels will not assemble on the PET (Scheme 3.2). Therefore, for these studies, a surface that is functionalized with a cationic species was chosen to drive anionic microgels to the surface by Coulombic attraction (Scheme 3.2). Unfortunately, attempts at generating a cationic PET surface were unsuccessful. As a result, a different substrate was chosen so that a more common functionalization technique could be employed, such as silanization chemistry. The studies shown here are on a glass substrate that was functionalized with a primary amine silane, but the use of silanization for surface functionalization can also be applied to silicone and silicon surfaces, which will be discussed in Chapter 5 and Appendix A.
Using silanization to modify the surface of a substrate also eliminated the drawback of using harsh plasma etching that can damage the original biomaterial. This advantage is utilized in Chapter 5 and Appendix A for maintaining the original integrity of soft elastomeric silicone substrates. Lastly, the utilization of spin-coating as the microgel deposition method, which is time-consuming and wasteful, was resolved using a rapid centrifugal deposition method, which is described in detail in Chapter 4.

Previously we have studied the interaction of cells with non-fouling microgel films that are spin-coated onto amine-functionalized glass. The particular cells used in these past studies were fibroblasts, which are cells that play a critical role in wound healing and form connective tissue. A more relevant cell-type to study in terms of controlled cellular adhesion during the wound-healing process are macrophages, which are one of the primary mediators of the inflammatory and wound healing processes, and are therefore an applicable cell to study in terms of their adhesion to a material’s surface during the foreign body response. Macrophages are considered to be more aggressive cells than fibroblasts because they are extremely adhesive to surfaces, and adhesion of this cell-type was also of primary focus in the microgel-coated PET studies. In conjunction with evaluating the macrophage-resistant behavior of non-fouling microgels deposited on glass, it was also necessary to interrogate the effect of using centrifugal
deposition (technique discussed further in Chapter 4) on the ability of the film to resist cellular adhesion. After several unsuccessful attempts at using centrifugally-deposited non-fouling microgel monolayers to prevent macrophage adhesion to amine-functionalized glass (data not shown), investigations were focused on evaluating the presence of microgels after incubation with biological media. Following incubation with macrophages, non-fouling microgel monolayers were no longer present on the surface, as can be seen in the Figure 3.1a. In addition, only a few patches of microgels remained on the surface when microgel monolayers were incubated in serum at 37 °C without cells (Figure 3.1b). Therefore, incubation with both serum and macrophages, at 37 °C, seem to result in complete removal of microgels from the surface. These results are surprising in light of the inability to remove non-fouling microgel monolayers from glass with an adhesive (tape) or high salt concentrations (1 M) at room temperature. The acid-containing microgels interact with the amine-functionalized glass by Coulombic attraction, and while this interaction is not appreciably destabilized by the addition of high salt solutions, a more complex environment containing lipids, proteins, and high amounts of salts, in combination with an elevated temperature, must significantly contribute to a weakened microgel-surface interaction. A similar weakened microgel-substrate Coulombic attraction was also observed for degradable microgels that were incubated at 37 °C in a high salt-containing buffer (Chapter 6). Furthermore, the presence of macrophages that are attempting to adhere to the surface can further contribute to a weakening of the microgel-surface interaction, or perhaps even pull the microgels off the surface in an attempt to phagocytose the material. In order to strengthen the microgel-surface interaction, a covalent attachment strategy was chosen, which was also necessary for the microgel-coated PET experiments.\textsuperscript{20} Covalent attachment was accomplished using carbodimide coupling chemistry to form a stable amide bond between the acids on microgels and the amines on the surface of the
substrate. This covalent attachment significantly improved the stability of non-fouling microgel monolayer films (Figure 3.1c & d).

![Figure 3.1](image.png)

**Figure 3.1.** Representative AFM images of microgel monolayers after incubation with macrophages and serum (a) and incubation in serum supplemented with phosphate buffered saline without cells (b). Covalently-attached microgel monolayers after incubation with macrophages and serum (c) and incubation in serum supplemented with phosphate buffered saline without cells (d).

While covalent attachment of microgels to the surface was needed in order to provide a stable microgel film interface, macrophages continued to adhere to these surfaces in a highly inconsistent manner, and in general exhibited a spherical morphology that is indicative of poor spreading. It was hypothesized that cells may be adhering to the unprotected underlying substrate *between* the microgels in the monolayer, and therefore
the monolayer film coverage is insufficient for minimizing macrophage adhesion. To improve the overall microgel coverage of the surface, thus eradicating the ability for macrophages to adhere to the underlying substrate, microgel films were constructed in a multi-layer fashion. Microgel multi-layers were fabricated using a polyelectrolyte layer-by-layer approach of alternating anionic microgels and cationic linear polymer. The polyelectrolyte layer-by-layer approach was first employed by Decher et al.,\(^ {29}\) and was also first demonstrated by Serpe et al.\(^ {23}\) for a microgel film containing alternating layers of microgels and linear polymer. In Serpe’s work, poly(allylamine) hydrochloride (PAH) was used as the cationic “glue” between acid-containing microgel layers. Polymers containing primary amines are known to cause higher levels of cytotoxicity, due to cell membrane disruption, than polymers that have the same number of amines but contain quaternary amines (quaternary amines are stiffer and therefore cannot attach to the cell’s surface as well).\(^ {30}\) Therefore, the quaternary amine polydiallyldimethylammonium chloride (PDADMAC) was used as an alternative to PAH between microgel layers. The assembly of alternating layers of anionic microgels and cationic linear polymer can be seen in Figure 3.2. After assembly and covalent attachment of the microgel monolayer, PDADMAC was passively adsorbed to the film. In the past, we have observed that the adsorption of a polycation onto an anionic microgel film, under slightly basic conditions, leads to a condensed microgel network.\(^ {26}\) This condensation upon adsorption of PDADMAC to the microgels is also most likely what occurs for the non-fouling microgel films, which also results in a charge reversal to slightly positive. As a result, more layers can then be added by centrifugally depositing anionic microgels, followed by passively adsorbing polycationic linear polymer. By using a multi-layered approach, the film became more continuous and was able to effectively block the underlying substrate (as illustrated in Figure 3.2). The results in Figure 3.3 demonstrate the ability of actively deposited non-fouling microgel multi-layers to block macrophage adhesion.\(^ {31}\) Macrophages adhere and spread well on the positive control tissue culture polystyrene
Glass that was extensively cleaned also shows cellular adhesion and spreading with an approximately five-fold reduction in number of cells adhering compared to TCPS. However, four-layer microgel films, which were generated quickly using centrifugation deposition (see Chapter 4), showed significant blockage of macrophage adhesion with an over 200-fold and over 30-fold reduction in number of adherent cells compared to TCPS and cleaned glass respectively. Furthermore, the few cells that adhere to the surface of the microgel multi-layer films do not appear to be able to spread, and therefore it is speculated that these cells have found small defects in the film, which presumably could be blocked with additional layers.

Figure 3.2. Illustration of multi-layer film formation with atomic force microscopy images showing microgel monolayer (a), bilayer (b), three layers (c), and four layers (d).
Figure 3.3. Representative fluorescence microscopy images of fluorescently stained macrophages adhering on (a) tissue culture polystyrene (TCPS), (b) cleaned glass, and (c) 4 actively deposited layers of PEG cross-linked pNIPAm microgels. Scale bar represents 200 µm (a) and 100 µm (b & c). (d) Quantification of cellular adhesion from fluorescence microscopy images with error bars representing the standard error.

The original intention of the work discussed here was to assemble microgel films using an approach that would be conducive for non-fouling microgels containing cellular adhesion peptides. It has been shown that acid-containing non-fouling microgels can be deposited on a substrate at a physiologically relevant pH without using plasma etching. Additionally, centrifugal deposition, as opposed to spin-coating, enables microgel assembly in a rapid and non-wasteful fashion because many samples can be made in parallel, with much shorter deposition times, and the microgel concentration can be reduced 80-fold. Furthermore, it has been shown that these films are successful at significantly decreasing unwanted macrophage adhesion. To accomplish the next task of mitigating specific control over cellular adhesion, a common cellular adhesion amino acid sequence, RGD, was conjugated to acid-containing microgels using carbodiimide coupling chemistry. The scrambled sequence RGE was used as a negative control. The peptide-conjugated microgels were then centrifuged as the top, or fifth layer, on the multi-layered films. However, repeated cellular adhesion studies showed little difference in macrophage adhesion between RGD and RGE conjugated microgel films. In Figure 3.4, fluorescence microscopy images that are representative from a large film sample show continued macrophage adhesion resistance to non-fouling microgel films (a). However, films containing RGD (b) or RGE (c) both show a slight increase in cellular adhesion, but
no statistical difference between the two. This slight increase in cellular adhesion, regardless of peptide type, is unexpected and indicative of poor and unpredictable cellular adhesion behavior in response to the tethered peptides. Both of the conjugated peptides may have allowed for some non-specific protein adsorption by direct interaction of proteins with the peptide, which increased cellular adhesion. Additionally, because the increase in cellular adhesion is hardly statistically different than the microgel film containing no peptide, it is possible that the peptides are not accessible to the cells or too low in concentration. Studies by Bysell et al. have suggested that small cationic peptides (1 – 10 kDa) can become homogenously localized within anionic microgels, rather than on the periphery of the microgel.\textsuperscript{32-33} The peptides studied here are < 1 kDa, and incorporated in very small amounts (only 1 mol\% of available acids). It is possible that the peptides are localizing more within the interior of the microgel, and are therefore not accessible to cells.

**Figure 3.4.** Representative fluorescence microscopy images of fluorescently stained macrophages on four-layered non-fouling microgel films (a), four-layered non-fouling microgel films with RGD-microgels as fifth layer (b), four-layered non-fouling microgel films with RGE-microgels as fifth layer (c), cleaned glass (d), and tissue culture polystyrene (e). Scale bar represents 100 µm (a-d) and 200 µm (e).

Kimberley Clarke, an REU (Research Experience for Undergraduates) student, performed a proof-of-concept study to better understand the accessibility of ligands that are conjugated to acid-containing microgels. In these experiments, two microgel constructs were compared: core microgels that are co-polymerized with acrylic acid, and a core-shell construct where the acids are only co-polymerized within the shell. Core-shell microgels have been successfully synthesized previously where a polymeric shell
can be added to a core particle, with the core acting as a nucleation site, in a two-step synthesis.\textsuperscript{21} For the bioavailability experiments performed in this chapter, biotin hydrazide was conjugated to the core and core-shell microgels, and films were assembled into monolayers using centrifugal deposition. The microgel films were then incubated with large streptavidin-coated polystyrene beads (approximately 1 µm in diameter) at 4 °C for 24 hours to assess the differences in biotin accessibility when biotin is conjugated to a core microgel compared to being localized on the periphery of the microgel. Large polystyrene beads were employed so that the streptavidin could not penetrate into the interior of the microgel, which is more representative of cell membrane-bound receptor accessibility to the cellular adhesion peptides conjugated to microgels. Experiments were initially performed at low temperatures because the amount of time required for the streptavidin beads to locate the surface-bound biotin was unknown, and incubation at lower temperatures will preserve the activity of streptavidin for longer periods of time.

As can be seen in Figure 3.5, after 24 hours of incubation with streptavidin-coated beads at 4 °C, both non-biotinylated core and core-shell microgels show no bead attachment (a) and (b). For microgels that were conjugated with biotin, there is a large difference in the number of attached streptavidin-coated beads between the core microgels and the core-shell microgels. The biotinylated core microgels show few beads attached to the film (c), whereas the biotinylated core-shell microgel films have significantly more streptavidin coated beads (d). Therefore, it is presumed that biotin must not be accessible in the core microgel construct, and the core-shell construct significantly improves the accessibility of biotin to the streptavidin-coated beads.
Figure 3.5. Representative brightfield optical microscopy images of non-biotinylated core microgels (a), non-biotinylated core-shell microgels (b), biotinylated core microgels (c), and biotinylated core-shell microgels (d) after incubated with streptavidin coated beads for 24 hours at 4 °C. Scale bar = 5 µm.

Similar accessibility experiments involving biotinylated core-shell microgels and streptavidin-coated beads were performed on multi-layered microgel films. Four-layered films were assembled using core non-fouling microgels and PDADMAC, and the fifth layer contained the core-shell microgel conjugated with biotin. These films were then incubated at 37 °C for 24 hours with streptavidin-coated beads. However, all films contained similar amounts of surface-attached streptavidin beads, even for surfaces not containing biotin (data not shown), which may be due to non-specific adsorption of the hydrophobic polymeric beads. However, it has been previously demonstrated that non-fouling microgel multi-layers are effective at minimizing macrophage adhesion (Figure 3.3), and therefore, due to the observed increase in surface-bound streptavidin beads to
the biotinylated core-shell microgel monolayers, as opposed to the biotinylated core microgels, the core-shell microgel construct was employed for cellular adhesion peptide conjugation in an attempt to improve accessibility of the peptides to cells. The peptide-conjugated core-shell microgels were deposited onto a four-layer non-fouling background film that had been assembled on glass in the manner described above for a multi-layered system. A four-layer non-fouling film and film containing RGD-peptide core-shell microgels as the fifth layer can be seen in Figure 3.6. Compared to the four-layer film, the five-layer film appears to have a higher density of microgels on the surface. Therefore, presumably, the fifth layer that contains peptide-conjugated core-shell microgels should have been added to the film. A scrambled peptide sequence (RDG) was also conjugated to a separate batch of core-shell microgels, which were also deposited as the fifth layer onto four layers of non-fouling microgels, to serve as a negative control for cellular adhesion. It is expected that cellular adhesion would be significant on the RGD-containing surface, but not the RDG-containing surfaces or microgel film containing no peptides. The results of the cellular adhesion studies can be seen in Figure 3.7. While the base non-fouling film continues to significantly reduce cellular adhesion, once again (similar to the results in Figure 3.4) there is some cellular adhesion to the peptide-conjugated surfaces with no difference between the RGD and RDG containing surfaces. With an increase in the amount of conjugated peptide (10-fold increase), there is a slight increase in cellular adhesion, but once again no significant difference between RGD-conjugated and RDG-conjugated films. As mentioned previously, the little difference in cellular adhesion observed between microgel films containing the two conjugated peptide-types may be due to an increase in non-specific protein adsorption to the peptides. The following parallel studies may suggest an alternative to the origin of these observed yet unexpected results.
Figure 3.6. Representative atomic force microscopy of assembled films containing four-layers of non-fouling microgels (a), four-layers of non-fouling microgels with RGD-conjugated core-shell microgels as fifth layer (b). Scale bar = 5 µm.

Figure 3.7. Fluorescence microscopy of fluorescently stained macrophages on four-layer non-fouling microgel film (a & f), four-layer non-fouling microgel film with RGD-conjugated core-shell microgels as fifth layer (b & g), RDG-conjugated core/shell microgels as fifth layer (c & h), cleaned plain glass (d & i), and TCPS (e & j). Amount of peptide conjugated was 1 mol % (b & c) or 10 mol % (g & h) of available acids on shell of core-shell microgels. Scale bars represent 100 µm for a-d and f-i and 200 µm for e & j.

At the same time the experiments involving peptide-conjugated microgel films were being conducted, it became apparent that polyelectrolyte multi-layered films fabricated from microgels and linear polymer were dynamic materials that appeared to be able to rearrange once solvated, as discussed in greater detail in Chapter 5. When these
same thin film constructs are fabricated on a soft elastomeric substrate (PDMS), and then manually manipulated (e.g. bending or stretching) under dehydrated conditions, visible “breaks” or deformations appear in the film. Upon water addition, the film immediately re-assembles with no evidence of deformation remaining when interrogated at the submicron level. It was speculated that the same dynamic behavior may be occurring on the surface of a hard substrate as well, even with a lack of a damaging event, when the films are incubated in aqueous media. Four-layer non-fouling films were interrogated for rearrangement by atomic force microscopy immediately after assembly by centrifugal deposition (Figure 3.8a), and then incubated in 100 mM ionic strength phosphate buffered saline (PBS) overnight at room temperature. After incubation in buffer, the film is more homogeneous and planar (Figure 3.8b), perhaps reaching a state of equilibrium where the film is more neutral within the entire assembly instead of maintaining areas of positive and negative charge. Additionally, as will be seen in Chapter 4, microgels that are assembled using centrifugal deposition take on a higher polymer chain conformation compared to passively adsorbed microgel films. This may cause a “relaxation” of the film when it is allowed to “age” in buffer over long periods of time, which may be occurring in Figure 3.8. Because these films are possibly in constant motion and rearrangement, it is unknown whether peptides are accessible at the surface for cellular recognition and binding, even though they were deposited as the top-most layer, or are evenly distributed in the lateral direction within the film. It is feasible to imagine that the peptide-conjugated microgels could phase separate to become internalized and buried within the film, or even phase separate into clusters at the surface. Due to the observed morphological changes in these films (Figure 3.8), in conjunction with the self-healing and lateral reorganization behavior exhibited in Chapter 5, it is incredibly difficult, at this particular time, to draw conclusions regarding cellular adhesion to non-fouling surfaces containing peptide-conjugated microgels. The dynamic behavior of these microgel polyelectrolyte multi-layered assemblies is under investigation.
Figure 3.8. Four-layer non-fouling microgel films on glass after centrifugal deposition assembly (a) and after incubation in high ionic strength (100mM) phosphate buffered saline (b). Scale bars = 20 µm for large image and 5 µm for insets.

3.4 Conclusions & Outlook

Non-fouling microgels can be used in the development of microstructured hydrogel coatings for controlling cellular adhesion to biomaterials in vitro and the foreign body response to materials in vivo, and exploited for various other applications in which complexity, tunability, and uniformity is desired. The work shown here shows the necessary changes in the previous film deposition technique that were needed to generate a non-fouling interface that is capable of controlling cellular adhesion by containing cellular adhesion peptides. These changes centered on modifying the deposition technique to allow deposition under physiological conditions in a non-wasteful and practical manner that also did not significantly alter the intergrity of the original biomaterial. These changes resulted in microgel films that were constructed in a multi-layered polyelectrolyte fashion, and were observed to effectively block macrophage adhesion. A cellular adhesion peptide (RGD) was then conjugated to a non-fouling core-shell microgel, to be deposited as the top layer of the film, in an attempt to direct
adhesion of cells in a controlled manner. However, these films were later found to be
dynamic and capable of rearrangement, which is further explored in Chapter 5.
Therefore, though a more bioavailable microgel construct (core-shell) was used in these
experiments, it is difficult to predict the accessibility or distribution of the peptide-
conjugated microgels within the film due to the rearrangement of the multi-layered
assemblies over time. Such dynamic behavior could be useful for triggered or dynamic
display of ligands if the film could be engineered to exhibit better controlled in terms of
it’s dynamic behavior. Lastly, the overall film mobility may play a role in the non-
fouling behavior of the microgel polyelectrolyte multi-layered films.

3.5 References


CHAPTER 4

CENTRIFUGATION AS A TOOL FOR FILM ASSEMBLY

4.1 Introduction

The construction of polymeric thin films is a subject of significant industrial importance for drug delivery,\(^1\) wettability control,\(^2\) corrosion\(^3\) or cellular adhesion inhibition,\(^4\) as well as of fundamental interest. Over the past few decades, a number of fabrication techniques have been employed to form films from a variety of building blocks, and their versatility has been demonstrated. Whereas extensive research has been conducted in the use of linear polymers\(^5\)-\(^8\) and continuous hydrogel networks\(^9\)-\(^12\) as polymeric interfaces, recent investigations into the use of solvent-swollen polymer colloids, or microgels, have illustrated the utility of colloidal gels as building blocks.\(^13\)-\(^18\)

When the solvent is water, microgels are composed of a water-soluble polymer cross-linked into a contiguous network, with the diameters of the particles typically ranging from ~100 nm to many microns.\(^19\)-\(^21\) When stimulus-sensitive polymers are used (such as pH sensitive\(^22\) or thermoresponsive\(^23\)) to form microgels, those particles and the resulting films can then exhibit responsive behavior by undergoing a volume phase transition as a function of that stimulus.\(^13\) Given their growing importance in film formation, microgels have been used as building blocks in the construction of films with potential utility in drug release,\(^24\)-\(^25\) tunable microlenses,\(^26\)-\(^27\) colloidal crystals,\(^28\)-\(^30\) and non-fouling films.\(^31\)-\(^33\) These interfaces have been assembled using a variety of deposition techniques such as dip-coating,\(^34\)-\(^40\) spin-coating,\(^24\)-\(^25,31\)-\(^33,41\)-\(^42\) or solvent-evaporation.\(^16\),\(^29\)-\(^30\) In addition, different hierarchical structures have been accomplished by layer-by-layer assembly,\(^24\),\(^25,34,41\) binary particle mixtures,\(^39\) or phase-separation induced deposition.\(^16\)

One particularly important aspect of film formation is control over the deposited material. For biomedical coatings, for example, the hydrophobicity, morphology,
elasticity, and chemistry of a synthetic material’s surface can have a dramatic effect on cell phenotype and behavior. Furthermore, complete coverage of the underlying substrate is typically desired in order to ensure total control of cell adhesion, spreading, and proliferation. Hydrogel-based materials can be fabricated to possess characteristics that make them suitable as a biomaterial because their volume consists mostly of water when in an aqueous environment and they are highly tunable in terms of their mechanical properties and chemical composition. Additionally, in a particulate form, microgels enable further complexity by enabling the assembly of multi-functional interfaces, due to a mixture of various types of microgels that can simultaneously assemble on the same surface, along with additional interesting morphologies. It is this ability to easily tune and adjust an interface that makes microgels an appealing material for controlling and studying how proteins, cells, and tissues interact with a synthetic interface.

In this chapter, centrifugation is used as a film fabrication approach to assemble microgel films in a fast, efficient, and reproducible manner. Whereas centrifugation has been used beyond its traditional use in purification, such as in the preparation of liposomes, rapid patterning of cells, or high-speed fabrication of photonic microfluidics, to our knowledge little has been explored in using centrifugation as a polymer film deposition technique. In this work, the use of centrifugation to fabricate microgel-based films is demonstrated for the first time, and what effect this additional parameter or force may have on the assembly of microgel monolayers is explored. The initial hypothesis was that centrifugation would simply decrease the amount of time it would take to create a continuous and uniform monolayer, as compared to a passive process. However, upon further investigation, it was evident that using centrifugation (referred to herein as “active” deposition) to force the hydrated particles onto a hard substrate resulted in an assembly that had smaller and more closely packed particles than what could be ultimately obtained with simple microgel adsorption (“passive“ deposition). To evaluate the generality of this phenomenon, a model system consisting
of two microgel particles of different sizes was studied. In addition, possible mechanisms for the observed results were briefly explored. Lastly, the technique’s ability to construct rapid multi-layered polyelectrolyte layer-by-layer films for fabricating uniform coatings is illustrated. The use of active deposition for the fabrication of non-fouling microgel multi-layered films, and their effectiveness at reducing cellular adhesion, are discussed in Chapter 3.

4.2 Experimental

4.2.1 Materials

All reagents used in this chapter but not listed here are described in Section 3.2.1. The cross-linker N,N'-methylenebis(acrylamide) (BIS) was used as received.

4.2.2 Microgel Synthesis

Microgel (1) was synthesized using a total monomer concentration of 70 mM with a molar composition of 85% NIPAm, 5% BIS, and 10% AAc. Surfactant SDS was used at a concentration of 1 mM. All of these components were dissolved in 49 mL of deionized water and filtered through Whatman #2 filter paper in a vacuum filtration system. The aqueous solution was then transferred to a three-neck round bottom flask and purged with N₂ for approximately 1 hour while the solution was heated to 70 °C. The initiator APS (0.0114 g), used in a total final concentration of 1 mM, was dissolved in 1 mL of deionized water and added to initiate the polymerization. The reaction was allowed to proceed for 4 hours at 70 °C under a blanket of N₂.

Microgel (2) was synthesized using a total monomer concentration of 100 mM, with a molar composition of 88% NIPAm, 2% BIS, and 10% AAc. Surfactant SDS and initiator APS were used in concentrations of 0.17 mM and 1 mM, respectively. The remaining conditions of the synthesis were carried out in the same fashion as described for (1).
4.2.3 Microgel Characterization

Dynamic light scattering (DLS) was used as previously described, to measure the hydrodynamic radius and diffusion coefficient of synthesized particles. A Protein Solutions DynaPro equipped with a temperature controlled microsampler was used for these measurements. Light scattering data was collected at an interval of 10 seconds per reading with a photodiode detector fixed at 90° relative to the incident the laser light (783.9 nm). Dynamics Software was used to calculate the autocorrelation decay from the random fluctuations in scattered light intensity. This information was then used to determine the diffusion coefficient of the sample in solution, which correlates with the hydrodynamic radius of the particles using the Stokes-Einstein equation. Electrophoretic mobility measurements were performed with a Malvern Instruments Zetasizer. All measurements were conducted using a dilute solution of microgels in pH 7.4 phosphate buffer containing 100 mM ionic strength (PBS).

4.2.4 Film Preparation

For a detailed description of substrate preparation and functionalization, refer to section 3.2.4. For the centrifuged films studied in this chapter, films were centrifugally deposited at a maximum rotor speed of 2250 × g for a specific amount of time. Passively adsorbed microgel films were made by simply controlling the exposure time of the functionalized glass to the microgel solution.

4.2.5 Multi-Layer Assembly

A detailed description of microgel multi-layer assembly can be found in section 3.2.4. The thickness of the microgel multilayer film was determined by using a razor blade to scratch the surface of the film and expose the underlying substrate, and AFM was used to determine the height of the film relative to the substrate.
4.2.6 Atomic Force Microscopy Imaging and Analysis

For details on instrumentation, software, and the cantilevers used for AFM, refer to section 3.2.5. All images were taken in air under ambient conditions, and a setpoint ratio (setpoint amplitude/free oscillation amplitude) of ~0.75 was used to ensure reproducible mechanical interactions between the tip and sample.

Quantitative image analysis was performed to calculate the average particle footprint area on the glass surface. Briefly, an iterative inverse mask was created to highlight the particles and the image was flattened to the 2nd order. A histogram was then generated to evaluate the bimodal distribution of surface height and particle height. Three times the standard deviation of the surface height was added to the average height of the surface to account for variations in the surface around the particles. A new inverted mask was generated based on this calculation, and the percentage of the image that was masked was calculated. This percentage was divided by the number of particles (counted manually) to give an average particle footprint area. The radial distribution function for the images was calculated using code written in-house in the IDL v.6.1 programming environment.

4.3 Results and Discussion

To study assembled microgel monolayers using the active centrifugation deposition technique, two different anionic microgel particles composed of N-isopropylacrylamide (NIPAm), acrylic acid (AAc), and the cross-linker N,N'-methylenebis(acrylamide) (BIS) were synthesized; these particles were used in the Coulombically-driven assembly of microgel films onto cationic silane-modified glass substrates. The particles were characterized using dynamic and static light scattering, and atomic force microscopy; the results of these characterizations are summarized in Table 4.1. The hydrodynamic radius of particle (1) is roughly half the size of particle (2), due to differences in cross-linker density, total monomer concentration in the reaction, and
amount of surfactant used in the synthesis. Accordingly, the diffusion coefficient for (1) is twice as large as (2). Additionally, (2) has an approximately 6 times larger footprint area when passively adsorbed onto a surface, and is slightly softer than (1), as evidenced by the higher $R_s/R_h$ (radius on the surface/radius in solution) ratio. However, both particles types have similar electrophoretic mobilities. Therefore, any differences seen under centrifugal deposition will be due to differences in sedimentation velocity, not the surface accessibility of anionic charges on the particle for attachment to the cationic substrate.

Centrifugal, or active, deposition is performed by placing the substrates of interest at the bottom of each well in a multiwell-plate (e.g. a cell culture plate), followed by the addition of a microgel dispersion into the well above the substrate. The plates are then placed in a swinging bucket, well-plate rotor. When the rotor spins, the well-plates swing out so as to align the centrifugal force ($g$), perpendicular to the plane of each substrate, thereby forcing the colloidal particles onto the substrate. For all experiments described in this study, the maximum centrifugal force of the rotor ($2,250 \times g$) was used. Because of its utility in the deposition of films on multiple samples simultaneously, centrifugal film deposition is fast, reproducible, and many samples can be made in parallel with a high degree of quality control.
Table 4.1. Microgel Characteristics. [a] Determined by dynamic light scattering in pH 7.4 phosphate buffer containing 100 mM ionic strength (PBS) at room temperature. [b] Determined by atomic force microscopy on 30 minute passively deposited samples. [c] Determined by electrophoretic light scattering in PBS at room temperature.

<table>
<thead>
<tr>
<th>Microgel</th>
<th>Hydrodynamic Radius, $R_h$ (nm)</th>
<th>Particle Footprint Area (nm$^2$)</th>
<th>$R_s/R_h$</th>
<th>Diffusion Coefficient (cm$^2$/s)</th>
<th>Electrophoretic Mobility ($\mu$m • cm/V • s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$172 \pm 0.1$ (15 $\pm$ 5% PD)</td>
<td>$9.4 \times 10^4 \pm 2 \times 10^3$</td>
<td>$1.01$</td>
<td>$13.4 \pm 0.4 \times 10^{-9}$</td>
<td>$-1.0$</td>
</tr>
<tr>
<td>2</td>
<td>$365 \pm 4$ (14 $\pm$ 0.3% PD)</td>
<td>$5.7 \times 10^3 \pm 1 \times 10^4$</td>
<td>$1.17$</td>
<td>$6.4 \pm 0.1 \times 10^{-9}$</td>
<td>$-1.06$</td>
</tr>
</tbody>
</table>

Microgels (1) and (2) were both subject to active film deposition, with passive deposition being used as a comparison throughout this entire study. Atomic force microscopy has been used previously to study passively adsorbed microgel particle monolayers, and those studies clearly illustrated the utility of the technique in this domain. It is important to point out that the microgels were dispersed and deposited in a phosphate buffered saline solution of high ionic strength (100 mM); these conditions have previously been shown to be appropriate for reducing anionic repulsion between microgels during film formation. Initial observations illustrate that microgels that are actively deposited form films that are fundamentally different than those deposited passively. As can be seen in Figure 4.1, when active deposition is used (Figure 4.1b and 4.1d), the microgels appear to be smaller and more closely packed than those deposited passively (Figure 4.1a and 4.1c). This difference in particle size is perhaps more clearly seen when smaller scan sizes are used (Figure 4.1 insets). It is also worth noting that regardless of the deposition method, the particles all have heights of only ~10-15 nm in the dehydrated state, illustrating the extremely low polymer density of the particles, which has been described previously. The calculated radial distribution functions for these images, also shown in Figure 4.1, quantitatively illustrate the differences in particle
spacing and nearest neighbor probability. Both microgel (1) and microgel (2) show nearest neighbor distances that are closer under active deposition, thus confirming that these films are more tightly packed when actively deposited as compared to passive adsorption. Interestingly, one might expect that microgels would flatten under centrifugal force and therefore result in particles with larger footprint areas and a film with a decreased particle density. However, the opposite is apparent in these experiments, which likely indicates that the particles have some lateral mobility to on the surface. The exact origin of this phenomenon is unclear, but it is likely that the presence of salt in the medium acts to screen the Coulombic interactions between the surface and the microgels, which would decrease the microgel-surface affinity.

![Figure 4.1](image)

**Figure 4.1.** Atomic force microscopy of microgels (1) (a and b) and (2) (c and d) under passive deposition (a and c) and active centrifugal deposition (b and d). Inset scale bars are 0.5 µm (a and b) and 1 µm (c and d). On the right, radial distribution functions are shown for each particle type and deposition condition, illustrating the quantitative differences in particle spacing between active and passive deposition.

The evolution of the microgel films was monitored as a function of deposition time under both active and passive conditions (**Figure 4.2**). The larger microgels (2)
appeared to reach full coverage faster than the smaller microgels (1) when centrifuged onto a surface. This is expected due to larger or more massive particles having a higher sedimentation velocity. The opposite was true for the passively adsorbed microgels, as the smaller particles have a higher diffusion coefficient and therefore reach the surface faster, which should result in faster coverage assuming an equivalent sticking probability. In addition, the particle footprint area was monitored as a function of time. Under both deposition conditions and for both microgel types, the microgel footprints appeared to decrease during the deposition. However, under active deposition, the microgels appear to shrink to smaller footprints than those obtained via passive deposition, and they also appear to shrink over a range of the deposition time. It is important to consider the possibility that tip convolution could be an issue when imaging particles that are spaced close together, which would result in microgels that would appear smaller in the x, y, and z dimensions. However, the data suggest that the AFM tip is effectively reaching the underlying substrate, as no loss in microgel height is observed as the particle spacing is decreased (Figures 4.3 and 4.4). An illustration drawn to scale of the particle dimensions with respect to the AFM tip, which also suggests that tip convolution should not introduce significant error in the analyses since the particle heights are so small relative to the tip dimensions, is also provided (Figure 4.5). There are a few possible mechanisms by which active deposition might result in smaller microgels that pack more tightly. For example, centrifugation could cause the microgels to concentrate in the solution above the substrate, and thus deswell due to an increase in the local osmotic pressure.49-51 Alternatively, the use of a high ionic strength buffer (100 mM) during deposition could permit the particles to desorb or rearrange on the surface, thereby dynamically reconfiguring the interface as microgels continue to strike the interface at a high velocity. Finally, it may be the case that actively deposited microgels have somewhat different adsorption/adhesion characteristics than those of passively deposited microgels. It is worthwhile noting that while the particle sizes decrease during deposition, the final
footprints do not approach the fully collapsed or dehydrated state of the microgels, which would be 2-4 fold smaller than that observed here. Thus, the particle footprints are more representative of the swollen particles, with some size decrease due to an additional factor such as those suggested above.

**Figure 4.2.** Microgel footprint area as a function of deposition time for centrifugation (active) and passive deposition techniques. (a) Microgel (1) and (b) microgel (2). *Denotes formation of a full monolayer. Error bars represent the standard error of the mean particle footprint area taken from three 10 µm x 10 µm (a) or 20 µm x 20 µm for (b) atomic force microscopy images.
Figure 4.3. Microgel (1) deposited over time passively for 30 min (a & b), 16 hrs PA (c & d), centrifuged for 30 sec (e & f), centrifuged for 2 min (g & h), and centrifuged for 10 min (i & j).
Figure 4.4. Microgel (2) deposited over time passively for 30min (a & b), 16 hrs PA (c & d), centrifuged for 30 sec (e & f), centrifuged for 2 min (g & h), and centrifuged for 10 min (i & j).
Figure 4.5. Illustration showing relative size of AFM tip to microgel film sample, and how the real sample compares to the data output from the AFM scan.

Considering these possibilities, it is unlikely that microgel pre-concentration during centrifugation is exclusively responsible for these observations, as small particle footprints might be expected early in the deposition, as well. However, Figure 4.2 clearly shows the microgels are initially larger and grow smaller over time during film assembly. Therefore, a dynamic rearrangement at the interface must be occurring during the formation of the film, both under active and passive deposition conditions, with the effect persisting even after formation of a high-coverage monolayer under active conditions. To further explore this phenomenon, we used a two-step deposition method, the results of which are shown in Figure 4.6. In this experiment, microgels were first adsorbed passively to obtain partial coverage (Figure 4.6a) or to obtain a higher coverage (Figure 4.6b), and then without replacing microgels or removing the film from solution, the samples were subjected to active (centrifugal) microgel deposition conditions. The results
illustrate that under these conditions, the average particle footprint area does still decrease upon active deposition, but not to the same extent as that observed during active deposition alone. Additionally, if particles are first passively adsorbed until a high coverage monolayer is generated, additional centrifugation does not induce a statistically meaningful change in the adsorbed microgel size. These data suggest that the passively adsorbed microgels do not rearrange dramatically or desorb from the surface during further active deposition. When space is available for microgels to deposit (Figure 4.6a), it appears that lateral repulsion between microgels might result in some decrease in microgel footprint and particle rearrangement. However, when microgels centrifuged on top of the passively deposited particles are unable to make their way onto the substrate (Figure 4.6b), significant restructuring of the interface is not observed. These results are in agreement with a previous study using similar pNIPAm-co-AAc microgels adsorbed on an amine-modified surface, where the authors illustrated that even with increasing NaCl concentrations there was no evidence of particle desorption from the surface.35 Considering these results, it is therefore likely that particle adsorption is fundamentally different between the two cases. Passive adsorption likely results in a polymer chain conformation that is closer to the thermodynamic minimum, whereas the active approach results in a polymer conformation that is higher in energy (kinetically determined) and is therefore more likely to evolve and age during centrifugation. This theory is further reiterated by observing the impact of ionic strength of the dispersion buffer on film assembly. When microgels are dispersed in a lower ionic strength buffer (2 mM), there is also a noticeable difference in microgel size and spacing (Figure 4.7). Though the particles are not as small and highly packed as under high ionic strength (100 mM) conditions, due to reduced shielding of repulsive side chains, active deposition once again results in a smaller size and spacing of microgels on the surface compared to passive deposition. This observation suggests that, even when shielding of the anionic side chains is significantly reduced, centrifugation can still overcome particle-particle repulsion to a
greater extent than a dip-coating method, presumably due to the higher energy used in deposition.

Figure 4.6. Atomic force microscopy characterization of the two-step deposition process for microgel (2). (a) A sub-monolayer was passively deposited first and then subjected to centrifugal deposition. (b) A full monolayer was first deposited passively and then subjected to centrifugal deposition. (c) Shows a full monolayer deposited actively for comparison. Scale bar is 1 µm. (d) Bar graph showing the average particle footprint area for all samples. Error bars represent the standard error of the mean particle footprint area taken from three 20 µm x 20 µm AFM images.
The recent work of FitzGerald et al.\textsuperscript{40} seems to support the hypothesis that particles can reorganize laterally due to interparticle repulsion. These investigators studied the passive adsorption of pH sensitive microgel particles using in liquid AFM.
imaging. Their results demonstrate the dynamic nature of such particles at the particle-surface interface. When these particles are in their non-deformable latex form at higher pH, they observe a significant deviation from what is expected from the random sequential adsorption model of hard spheres, where the diameter of the particles at the interface is almost twice the size compared to their size in solution. Furthermore, when the pH was adjusted to more acidic conditions, thus inducing a latex to swollen microgel transition, this swelling pressure caused neighboring particles to desorb from the surface. Even though Coulombic interactions exist between the particle and substrate, the particle-particle interactions dominate in their example. These results illustrate an extreme case of dynamic microgel adsorption where lateral particle interactions can dictate particle coverage. Similarly, the results demonstrated in this chapter illustrate that polymeric particles can undergo size changes and film rearrangement as a function of coverage, albeit in the limit of strong microgel-surface interactions.

Multi-layered polyelectrolyte microgel interfaces, which has been demonstrated previously,

were fabricated using rapid centrifugal film deposition to illustrate the utility of the technique. The progression of layers of a multi-layered microgel film is shown in **Figure 4.8**. Atop a glass substrate that was rendered cationic by amine functionalization, anionic microgels (Microgel 2) were alternatively layered with poly(diallyldimethylammonium chloride), or PDADMAC, as a cationic polymer. As more layers are added (up to four layers of microgels) less of the underlying glass substrate appears to be exposed and the resulting film is quite uniform. The average film height for the 4-layer film is ~60 nm in the dry state, as determined by AFM line profiles across a scratch in the film introduced by a clean razor blade. Previously, it has been shown that spin-coating of microgels onto a substrate provided the high coverage needed to block the background substrate from protein and cellular adhesion. However, spin coating is an intrinsically serial process that can be quite wasteful of material. However,
as shown below, centrifugation-based assembly can be parallelized and also provides the required high coverages using a rapid multi-layering approach in a non-wasteful manner.

Figure 4.8. AFM images of microgel thin films constructed in a multi-layered fashion (using microgel 2). Each image has a 20 µm x 20 µm scan size with a 5 µm x 5 µm inset. Images were obtained from 1, 2, 3, and 4 layer films (left to right) formed by active deposition.

4.4 Conclusion

Centrifugation has been demonstrated to be a rapid and robust method for generating colloidal films. When using hydrated anionic microgel particles to construct an interface, centrifugation deposition results in particles that are smaller and more closely packed compared to a more common dip-coating, or passive adsorption technique. The footprint area of centrifuged particles actively shrinks during the course of assembly. Passively deposited microgels appear to stop decreasing in footprint size at an earlier stage in the adsorption process, and when these films are subsequently subjected to centrifugally forced microgel deposition, the centrifugation approach does not force significant morphological changes in the deposited particles. The impact of this phenomenon on particle adhesion, modulus, film stability or “aging” (see Chapters 3 and 5) is currently being explored. In addition, it has been demonstrated that the centrifugal deposition approach enables a functional multi-layered microgel interface with a high degree of uniformity and substrate coverage, and became an important tool for rapid assembly of non-fouling microgel films for reduced cellular adhesion to substrates (see
Chapter 3). These interfaces can be used in the development of microstructured hydrogel coatings exploited for various applications in which complexity, tunability and uniformity is desired.

4.5 References


CHAPTER 5

SELF-HEALING MULTILAYERED FILMS

5.1 Introduction to Self-Healing Materials

Self-healing materials have the ability to undergo repair following a damaging event. Over the past few decades there has been a growing interest in materials that can self-heal, which can increase a material’s lifetime, reduce replacement costs, and improve product safety. Self-healing systems can be made from a variety of materials, but polymers have been extensively explored due to their chemical and mechanical tunability, and the ability to create dynamic materials.\textsuperscript{1-4} Most materials do not have the inherent ability to heal themselves, typically because their building blocks are organized into rigid architectures and therefore cannot migrate across defects longer than the molecular length-scale, or because the molecular components are not chemically labile enough to re-form bonds following rupture. In fact, most materials suffer from both problems. However, several groups have developed approaches to solve these issues. Materials that contain reversible chemistries or weak interactions within the polymer matrix can successfully be mended back together following introduction of a defect.\textsuperscript{5-7} This approach is limited to particular chemistries and oftentimes the residual “dangling chains” will interact with other chains on a single side of the gap, as opposed to cross-gap interaction, which prevents healing if the material is not mechanically re-connected soon after cutting. Another approach involves heating the polymer above its glass transition temperature ($T_g$), thereby increasing the mobility of the chains, causing rearrangement and molecular interdiffusion to promote “crack healing”.\textsuperscript{8-10} The obvious limitation to this approach is the need for the external application of heat, meaning that truly autonomous healing is not possible. Other demonstrations involve filling of a void by the release of healing agents or inhibitors into cracks,\textsuperscript{11-14} or by an induced phase separation
of nanoparticles towards damaged areas.\textsuperscript{15} However, the embedded reservoirs or nanoparticles are incorporated in a limited number, and therefore it is unlikely that such materials could continue to heal after recurrent damage in the same area.

The vast majority of healing processes that have been studied involve robust polymeric structures such as epoxy coatings\textsuperscript{5,11-12} or elastomers,\textsuperscript{6} but more delicate architectures such as hydrogel thin films have not yet been studied as self-healing materials that can heal induced mechanical damage. In this chapter, the autonomic self-healing behavior of polymeric thin films assembled from colloidal hydrogel building blocks is explored. Hydrogels have been a topic of growing interest over the past twenty years due to their unique properties and the wide variety of applications in which they can be exploited,\textsuperscript{16-18} and therefore the ability of hydrogels to “self-heal”, as demonstrated here, offers an additional useful property of hydrogel-based films. The films described here are fabricated via Layer-by-Layer (LbL) polyelectrolyte self-assembly, as discussed in \textbf{Chapters 3} and \textbf{4}, and \textbf{Appendix A}, on a soft elastomeric substrate (PDMS). After mechanically induced damage, these films are able to undergo rapid (<seconds) healing of many-micron sized defects that span the entire coated area (1 cm\textsuperscript{2}), upon exposure to water.\textsuperscript{19} The self-healing properties displayed by these coatings enable the use of hydrated polymer films in applications where rough (e.g. surgical) handling and transient damage are inevitable, such as in biomedical implants, which became important for coating silicone breast implants (\textbf{Appendix A}). In this chapter, the self-healing behavior of multi-layered polyelectrolyte films is interrogated by altering aspects of the film (soft microgel vs. rigid sphere, different microgel types, different molecular weight polycations, and different number of microgel layers), and by photobleaching fluorescently-labeled microgel multi-layered films to observe local microgel rearrangement.
5.2 Experimental

5.2.1 Materials

All reagents used in this chapter but not listed here are described in Section 3.2.1. PDADMAC of MW = 100,000 – 200,000 (100-200K) and MW = 40,000 (40K) were purchased from Sigma Aldrich. Poly(dimethylsiloxane) (PDMS) Sylgard 184 was purchased from Dow Corning in a two-part system consisting of elastomeric base and curing agent. The fluorescent monomer 4-acrylamidofluorescein (AFA) was synthesized by Michael Serpe\(^{20}\) using a procedure described elsewhere.\(^{21}\) Carboxylated-modified polystyrene spheres (1 µm diameter) were purchased from Polysciences.

5.2.2 Microgel Synthesis & Characterization

Synthesis of non-fouling microgels used this chapter are described in Section 3.2.2. Instead of a 50 mL synthesis, 100 mL syntheses were performed. Dynamic light scattering (DLS) was used to determine the hydrodynamic radius of the non-fouling microgels. A more detailed description of DLS can be found in Section 1.3.2.2 and 4.2.3. Phosphate buffer pH 7.4 and formate buffer pH 3 were used as the dispersion medium for the measurement. \(^1\)H NMR was used to verify the incorporation of PEGDA575 cross-linker using D\(_2\)O as the solvent.

Two different sets of BIS cross-linked microgels were used in this chapter. The microgels used in Figure 5.7 were synthesized following the procedure in Section 4.2.2 for microgel (2). The second set of BIS cross-linked microgels used for the quantitative experiments were synthesized in the same manner as described in Section 4.2.2 except using the following molar compositions: 68% NIPAm, 2% BIS and 30% AAc. Surfactant (SDS) and initiator (APS) were both used in concentrations of 1 mM. DLS was also used to characterize the size of the BIS cross-linked microgels when dispersed in PBS buffer containing 100mM ionic strength.
Microgels that were fluorescently labeled were synthesized in the same manner as described in **Section 4.2.2**, except using the following molar compositions: 0.1% 4-acrylamidofluorescein (AFA), 30% AAc, and 69.9% NIPAm. AFA was dissolved in DMSO and added to the warm oxygen purged synthesis 10 minutes prior to initiation. Surfactant (SDS) and initiator (APS) were used in concentrations of 1 mM. Once again, DLS was used to characterize the hydrodynamic radius when dispersed in PBS containing 100 mM ionic strength.

### 5.2.3 Substrate Preparation & Microgel Film Assembly

PDMS was prepared by mixing a 1:10 ratio, by weight, of curing agent and elastomeric base. After sufficient mixing in a plastic petri dish, the PDMS was covered and placed in a vacuum chamber to remove air bubbles for approximately 15 min. The material was then allowed to cure in a 50 °C oven for 24 hours. Using a razor blade, the PDMS was then cut into 1 cm x 1 cm squares (for the non-fouling films) or 9 mm x 9 mm squares (for the BIS cross-linked microgel films), which were 1 mm in thickness, and washed in hexane until the PDMS squares stopped swelling (approximately 2 hours), to ensure the removal of any uncured material. Afterwards, the PDMS pieces were removed from hexane and placed in a 50 °C vacuum oven overnight to remove residual solvent. The PDMS pieces were rinsed with ethanol, deionized water, and then placed in a plastic eppendorf tube and lodged in such a way so that they would remain submerged (PDMS will normally float in aqueous solutions). The squares were incubated in water for 30 minutes, and then the water was replaced with a 1.2 M aqueous solution of hydrochloric acid overnight. Afterwards, the PDMS was rinsed with water by removal and replacement of water directly into the eppendorf tubes three times. The PDMS was then rinsed with absolute ethanol and then equilibrated in absolute ethanol for 30 minutes. The ethanol was replaced with a 1 % by volume solution of APTMS in absolute ethanol and allowed to interact with the PDMS for 2 hours. Functionalized PDMS was
then rinsed with 70% aqueous ethanol and placed in a well-plate with 20 mM phosphate buffered saline (100 mM ionic strength). During incubation in buffer, the PDMS become turbid. After 30 minutes, the buffer was replaced with a 0.1 mg/mL solution of microgels dispersed in the same buffer. Microgels were centrifugally deposited, covalently attached, and assembled in multi-layers using the procedure in Section 3.2.4. All PDADMAC solutions were kept at a constant total monomer concentration of 0.1 monoM in PBS (100mM ionic strength).

5.2.4 Film Characterization

Films were characterized using four imaging methods. A FujiFilm FinePix J20 camera with a 10 megapixel CCD chip was used to capture unmagnified images. Microgel films were also imaged using atomic force microscopy and brightfield optical microscopy. For specifics pertaining to AFM and optical microscopy, refer to Sections 3.2.5. Brightfield optical microscopy was performed on an Olympus IX-70 inverted microscope equipped with a Cooke Corporation PixelFly black and white CCD camera. For fluorescence microscopy of AFA-labeled microgels, the same Olympus IX-70 inverted microscope was used, equipped with a Coolsnap CCD camera (RS Photometrics). For all images taken either in brightfield optical microscopy or fluorescence microscopy, the 1.5x slider was used for all objectives.

5.2.5 Quantitative Stretching and “Break” Frequency Analysis

Films were stretched using a in-house-built apparatus equipped with a translational stage (see Figure 5.1). This allowed for precise control over stretching of the microgel-coated PDMS samples. Each sample was place in the apparatus, with the microgel film facing up, by first placing one side of the sample onto a clean glass slide (on the long side) and another glass slide was placed on top to sandwich the film in between the two slides. In this manner, approximately 1 mm of the sample was clamped
between the glass slides, with the rest of the sample was exposed to air. Clamps were then used at the shorter ends of the glass slide to clamp the sandwich construct together while it was placed in between two washers in the apparatus. Two glass slides were taped together and placed between the two washers on the opposite side so that the sample would remain horizontal while it was being tightened down with a bolt on top of the washers. After the taped glass slides and glass slides sandwiching the sample were tightened down so that they would not move, the translational stage knob was set to a “0” reference point. Opposite of the microgel coated PDMS sample, two glass slides were again used to sandwich the other side of the sample (about 1 mm) and kept straight upon tightening in the same manner as described above with two taped glass slides. The sample was visibly interrogated to ensure that it was flat (parallel to the ground), and not bent or stretched. Water was then added to the film to heal it of any damage that may have occurred during setup in the apparatus. Kimwipes were used to draw liquid away from the sides and any remnant water that would not leave the sample in this manner was allowed to air dry. Films were stretched by turning the translational stage knob by 1 mm, and the bolts were unscrewed on one side to allow the film to retract. Stretched films were then carefully removed from the glass slides and placed on a glass coverslip for imaging by brightfield optical microscopy. Each sample was imaged at least three times in the center of the stretched region. Each sample was interrogated twice in this manner with a water heal in between measurements. Two samples were fabricated for each of the 400-500K and 100-200K PDADMAC films containing the different number of layers, and interrogated by two trials each. Only one sample was completed for each of the 40K PDADMAC films containing the different number of layers, with two trials performed for each (the second sample set showed no breaks by optical microscopy).
Figure 5.1. Stretching apparatus containing a translational stage and knob (a) that moves the left side of the setup (b) in the horizontal direction when the knob is turned. The other side of the apparatus is fixed (c). The sample is suspended in the air and sandwiched between two glass slides on either side (d).

The stretching images were analyzed using Igor Pro 5.04B. After the images were loaded into Igor, the image processing analysis package was used to draw 1,000 line profiles across the image, and generate an average line profile from these 1,000 lines. The Fourier transform of the average line profile data was performed to convert the data to amplitude vs. frequency. A power spectral density (PSD) function was then applied to this data to convert the Fourier transform into a histogram plot that shows the most probably frequency in the line profile data.

For the films containing 400-500K or 100-200K PDADMAC, each film break frequency is an average of the frequency measurements from all the images taken for each sample between the two trials, and the error bars represent the standard deviation of the average between the two different film samples. For the film containing 40K PDADMAC, only one sample was completed for each film containing the different number of layers. The average represents the average of the frequency measurements images taken between two stretching trials, and the error bars represent the standard deviation between the two trials.
5.2.6 Film Thickness Measurements

For measuring the thickness of the microgel films, microgels were assembled on amine-silanized glass in the same manner described in Section 3.2.4. After microgels were assembled on glass, the samples were immediately rinsed with deionized water and blown dry with \( \text{N}_2 \). Using a clean and sharp razor, films were scratched by applying gentle pressure while scratching. Films were then interrogated using AFM. Instead of the height data channel, the z-sensor data channel, which is less susceptible to imaging artifacts, was used to determine the height of the film. A histogram plane fit in the x-direction was applied to the z-sensor image to eliminate the tilt gradient. The histogram flattening takes a histogram of every scan line, determines the “background” substrate, and offsets the data so that the value of this background is constant from line to line. A height line profile was drawn across an area containing the microgel film, and an average of the highest point and the lowest point was calculated to obtain the average film height in the image. The difference between the film height and the height of the surface within the image was used to determine the film thickness. The film thickness data represent an average film thickness from three different scans that were analyzed in the manner described above, and the error bars are the standard deviation of those three measurements.

5.3 Results and Discussion

The microgels used in the first set of studies in this chapter are composed of \( N \)-isopropylacrylamide (pNIPAm), acrylic acid (AAc), and the cross-linker poly(ethylene glycol) diacrylate (MW=575) (PEGDA575). Their hydrodynamic radius is 277 ± 25 nm and 510 ± 31 nm in pH 3 and pH 7.4, respectively. Their dehydrated height on a glass surface is approximately 60 nm. These are similar to the non-fouling microgels used in Chapters 3 & Appendix A, and were employed for these studies since they are relevant to the group’s efforts in non-fouling biomaterials coatings. Similar microgels, when used
to coat an implantable biomaterial, have shown to dramatically reduce both protein and cellular adhesion \textit{in vitro},$^{22-23}$ and reduce leukocyte recruitment, cytokine release, and fibrous capsule thickness \textit{in vivo} (Chapter 2).$^{24-25}$ These previous studies illustrated the effectiveness of using non-fouling microgels as a coating for reducing the foreign body response, which can ultimately improve the performance and lifetime of implantable biomedical devices. Importantly, we now show that such coatings can likely withstand the rigors of surgical handling and should autonomously heal any defects associated with the act of implantation.

Previous studies in the use of non-fouling microgel multi-layered films suggested dynamic microgel reorganization within the films, both on hard substrates (Chapter 3) and soft substrates (Appendix A). Whereas these observations were not quantitatively explored at that time, they suggested the potential for defect healing properties. Thus, to more precisely investigate the response of microgel multi-layers to controlled damage, the films were deposited on an elastomeric substrate, poly(dimethylsiloxane) (PDMS), which allowed for the controlled mechanical manipulation of the substrate and its associated microgel coating. PDMS was functionalized using hydrochloric acid (generates surface silanols) and silanization with a primary amine silane, to which microgel films were assembled into four-microgel layers using centrifugal deposition of microgels (Chapter 4) alternating with passively adsorbed PDADMAC containing a molecular weight of 400,000–500,000 (400 – 500K). A representative film is shown in Figure 5.2(a), (b), and (c) at different magnifications. Panel (a) is a photograph of coated 1-mm thick PDMS on a supporting microscope coverslip, while panels (b) and (c) are brightfield microscopy and atomic force microscopy (AFM) images, respectively. At all magnifications, the films appear homogeneous, with the dominant roughness features (observed via AFM) arising from the microgel building blocks themselves.

Microgels have previously been used to fabricate 2D and 3D arrays on solid substrates via LbL assembly.$^{20,26-27}$ In those earlier studies, it became apparent that
cationic linear polymer was able to penetrate the microgels and strongly cross-link the anionic acidic side chains within the microgels. Subsequent addition of another microgel layer resulted in a three-dimensional, Coulombically cross-linked hydrogel network. These investigations led to an understanding of how linear polyelectrolytes can render individual microgels “sticky”, and also how the interplay of both strong and weak interactions impact the assembly and swelling properties of such materials.

Figure 5.2. Visualization of damage introduced by multiple “stabs” with a 5 µL pipette tip. Images were taken by (a, d, g) digital camera (scale bar = 2.5 mm), (b, e, h) brightfield optical microscopy (scale bar = 20 µm), and (c, f, i) atomic force microscopy (scale bar = 10 µm), before damage (a, b, c), after damage (d, e, f), and after healing by rehydration (g, h, i).

After assembly of the microgel film on PDMS, any physical contact with the coating appeared to change the film appearance dramatically. These observations were also made for microgel coatings on the silicone breast implant casing (Appendix A).
Although a razor blade is commonly used to induce damage to demonstrate self-healing, this approach was avoided to prevent irreversible damage to the PDMS. Therefore, an object with a blunt tip was used to illustrate the macroscopic healing properties. As can be seen in Figure 5.2 (d), (e), and (f), by simply pressing a 5 µL pipette tip into the surface, a ring remains on the film. The ring defect can be seen by eye, optical microscopy, and AFM, and renders deep grooves and ruffled regions in the film that are a few microns wide. During damage, it appears that the microgels are redistributed, which is apparent by the elevated regions (high microgel density) along the edges of the cracks (low microgel density). However, the addition of water to the film erases these defects without observable desorption of microgels from the film (Figure 5.2 (g), (h), and (i)). In fact, the defects heal so quickly (on a timescale of seconds), that direct microscopic observation of the healing process has not yet been possible. For example, the addition of water to the film necessitates manual refocusing of the optical microscope, the process of which takes longer than the defect healing time. Additionally, a high salt solution (1 M) and warm temperatures (50 ºC) do not prevent the rapid healing of these films and do not induce significant particle desorption. From these results, it is apparent that microgel-based films can survive extensive disturbances from an external object, and then undergo dramatic rearrangement back to the original structure.

A comparable study was also conducted on a four-layer film assembled with alternating layers of carboxylated polystyrene microspheres (1 µm diameter) and 400-500K PDADMAC (Figure 5.3). The rigid sphere multi-layers appear to form a homogenous and continuous film that completely covers the PDMS (Figure 5.3 (a), (b), & (c)). Once the film is poked with a pipette tip, in the same manner as the microgel film, there is a ring defect. The defect is shown as the bright areas in Figure 5.3 (d) & (e) due to a loss of particles that allows more light to transmit through the film. When water is added to the film (Figure 5.3 (f)), there is no self-healing of the rigid sphere structure, and particles start to visibly desorbed from the surface and float in the water
(denoted by red arrows in Figure 5.3 (f)). When the rigid sphere film is dried, the damage looks identical to how it appeared before water addition (Figure 5.3 (g)). It is also interesting to note that the damage that the rigid sphere film endures is very different than the microgel film damage. When the pipette tip is pressed onto a microgel film, the “ruffled” regions are the areas surrounding where the pipette tip touched the surface, and the film actually appears intact in the areas where it came into direct contact with the pipette tip (left bottom corner of image in Figure 5.2 (e)). The damaged areas in the polystyrene film, however, are exactly where the pipette tip came in contact with the film. When the microgel film is disturbed by an external object, perhaps the stress from the object causes strain on the underlying PDMS that transmits in all directions, away from where the object makes contact, to dissipate the stress. As the PDMS locally deforms from this stress, the microgel film atop these strained areas break in an attempt to also dissipate the stress. Once the stress from the pipette tip is removed, the elastic PDMS relaxes, but the plastic deformation in the microgel film remains until solvated to mend the broken film by microgel swelling and reorganization. The film is strong, however, because it maintains its overall integrity and microgels do not desorb. Conversely, once the rigid sphere multi-layer film is poked, the particles are immediately dislodged in the same area where the object made contact. In addition, deformations around the immediate points of contact are not seen because the rigid sphere film does not experience the stress induced by the underlying deformed PDMS, perhaps due to the lack of multiple points of contact of the spheres with the substrate. These comparisons illustrate how the lack of polyvalency between rigid anionic microsphere and the amine-functionalized substrate, in addition to a lack of interpenetration of the polycation within the sphere, which in turns reduces polyvalent interactions between microspheres, dramatically reduces the strength of the film. Microgels, however, can polyvalently attached to the amine-functionalized surface, and the polycationic polymer in microgel
can penetration the microgel, thus forming more polyvalent interactions that increases the strength of the interaction between microgels.

**Figure 5.3.** AFM of a four-layer carboxylated polystyrene microsphere film assembled with 400-500K PDADMAC (rigid sphere film) (a). Brightfield transmission microscopy of the rigid sphere film after assembly (b & c), poked with a pipette tip (d & e), immersed in water (f), and dried (g). Red arrows in (f) denote desorbed particles. Scale bars represent 80 µm (b, d, f & g) and 20 µm (c & e).

To interrogate how controlled mechanical stretching and bending of the material would affect the microgel coating, microgel multilayered coated PDMS pieces were stretched by 10% in length and bent to an angle of 90 degrees. **Figure 5.4** shows, via optical microscopy and AFM, the damage that occurs during these treatments. Stretching creates parallel breaks or cracks in the film that are perpendicular to the axis of stretching, whereas bending generates a 2D network of fractures. The inset images confirm the presence of the particulate film in the damaged region; the observed defects are present in the coating and not the underlying PDMS. Again upon the addition of water, the microgel multilayers recover back to their original continuous structure without any evidence of breaks in the film. It is important to point out that these defects do not occur on chemically treated and silanized PDMS alone (**Figure 5.5**), and are exclusively associated with the hydrogel coating.
Figure 5.4. Film damage introduced by (a) stretching and (b) bending deformation, as observed by brightfield optical microscopy (scale bar = 10 µm). Samples are shown after deformation (a, c) and after healing (b, d). During bending of the sample (c), the microgel film is present on the outer surface. Insets are 5 µm x 5 µm atomic force microscopy scans of the damaged or healed regions (scale bar = 1 µm).
Figure 5.5. PDMS that has been HCl-treated and APTMS-functionalized before (a) and after (b) stretching. Four microgel multilayers on HCl-treated and APTMS-functionalized PDMS before (c) and after (d) stretching.

In Figure 5.6, topographical changes are shown by line profiles drawn across the entire width of the AFM images. Defects as deep as 400 nm and as wide as 4 µm occur during mechanical manipulation. After each damage event, the microgel film recovers completely to its original topography. This process can be repeated on the same sample without the underlying PDMS ever becoming exposed. In other words, multiple damage/healing cycles do not result in de-lamination of the microgel film.
Figure 5.6. AFM scans (20 µm x 20 µm) of defects induced on the same sample before deformation and after healing in order from left to right (scale bar = 5 µm) (a). AFM line profiles of each scan drawn across the entire image are shown to illustrate the reversibility of the phenomenon (b). The border color for each scan in (a) corresponds to the color of each curve in (b).

The self-healing capabilities exhibited by microgel polyelectrolyte multi-layers are not limited to PEGDA575 cross-linked non-fouling microgels assembled with 400 – 500K PDADMAC. Four layered films fabricated from pNIPAm, AAc, and the cross-linker \(N,N'-\text{methylenebis}(\text{acrylamide})\) (BIS) also show the ability to undergo damaging and healing events. As can be seen in Figure 5.7, films fabricated from BIS cross-linked pNIPAm-co-AAc microgels can be stretched by 10% in length, show parallel “breaks” in the film similar to those seen for non-fouling microgel films (Figures 5.4 – 5.6), and heal once solvated with water (Figure 5.7 (c) & (f)). The multi-layered films also exhibit
damage and healing behavior when PDADMAC of different molecular weights are used (Figure 5.7). Interestingly, however, the damage appears to be dissimilar when PDADMAC of different molecular weights are utilized. As can be seen in Figure 5.7 (b) and (e), when the films are subject to the same amount of strain (stretch by 10% in length), the “breaks” that are exhibited throughout the film are spaced at different intervals. More specifically, the film containing the lower molecular weight PDADMAC has shorter distances between cracks than the higher molecular weight PDADMAC.

![Figure 5.7. AFM of four-layered microgel films using BIS cross-linked pNIPAM-co-AAc microgels and 400-500K PDADMAC (a - c) and 100-200K PDADMAC (d - f). Films were interrogated after assembly (a & d), after stretching by 10% in length (b & e), and after healing with water (c & f).](image)

To better understand what effect different PDADMAC molecular weights would have on the extent of damage on the microgel multi-layered films, BIS cross-linked pNIPAm-co-AAc microgels were assembled on PDMS using PDADMAC molecular weights of 400-500K, 100-200K, or 40K. The microgels used in these quantitative experiments had a hydrodynamic radius ($R_h$) of 304 (PD = 12%) when dispersed in phosphate buffered saline (PBS) containing 100 mM ionic strength. In Figure 5.8b-d,
representative AFM images of four-layered microgel films containing the different molecular weight polycations can be seen. The films all appear reasonably homogeneous and completely cover the underlying PDMS substrate. Compared to the monolayer film (a), all the multi-layered films contain microgels that are smaller, meaning they have been effectively subjected to polycation adsorption, which condenses the microgel network,\textsuperscript{26-27} and the lateral distance between microgels also appear similar.

![AFM images](image)

**Figure 5.8.** Atomic force microscopy height images of a microgel monolayer (a), multi-layered films containing four microgel layers with different Mw PDADMAC 400-500K (b), 100-200K (c), and 40K (d) assembled on functionalized PDMS. Scale bar represents 2 µm.

Films were stretched in a controlled manner using a custom-built stretching apparatus equipped with a translational stage, as described in Section 5.2.5. The films were all stretched (or strained) by the same amount (11 % by length), and this damage can be seen by brightfield optical microscopy (**Figure 5.9**). The film breaks appear farthest apart for the largest molecular weight PDADMAC, and become more closely spaced as the molecular weight of the polycation decreases. This may be indicative of a weaker and less cohesive multi-layered film as the size of the polycation between microgels decreases. Therefore, as the film is stretched, the lateral interactions break more readily, thus resulting in a film that breaks more often. Microgels in films that contain a larger molecular weight polycation can continue to slide past one another during the stretching event, while still maintaining overall cohesion within the film due to the longer PDADMAC, and then break at weak points in the film to dissipate the stress. In addition, the contrast of these breaks, relative to the rest of the film, also decreases as
the molecular weight of PDADMAC decreases. In the case of the largest molecular weight polycation, there are dark regions, light regions, and regions that are in between (or grey). The lightest regions may correspond to where the film breaks because more light can be transmitted through the film, and the darker regions may be areas where the microgels have rearranged and built up along the crack, thus allowing less light to travel through the film in these regions (the light and dark assignments may also be reversed due to light scattering). This is further illustrated in the AFM images shown previously when a film is stretched and exhibits cracks. For example, in Figure 5.7 (b), the regions right along the cracks are taller compared to areas of the film that contain microgels that are not disturbed. These taller features may be caused by a built up of microgels along the edges of the breaks within the film. Perhaps when the film breaks upon stretching, and the elastic recoil from the underlying PDMS brings the film back together when the stress is released, the edges of the microgel film breaks push up against one another when brought back together. The longer molecular polycation allows for more sliding before breaking, thus essentially elongating the film, which induces more build up at the edges when brought back together. It is also possible that some buckling of the underlying PDMS may be contributing to the strong topographical features seen in the damaged films.

Figure 5.9. Representative images from bright field transmission optical microscopy of microgel multi-layered films containing four microgel layers with PDADMAC 400-500K (a), 100-200K (b), and 40K (c). Scale bar represents 20 µm.
The results from the controlled stretching experiments can be seen in Figure 5.10. By taking the power spectra density (PSD) of the Fourier transformed bright field optical microscopy images, the most probably break frequency was found for each film. Both polycation molecular weight and the number of microgel layers in the film were altered to observe what effect these changes would have on the film break frequency. The trend remains the same; as the molecular weight of the polycation decreases, the distance between breaks in the film, or length per break cycle, also decreases. As the number of microgel layers is increased, the frequency of breaks does not appear to change by a statistically meaningful amount when the film is stretched. This observation seems logical considering the fact that microgel layers are added to the film in a direction that is orthogonal to the direction in which the stress is applied. An exception to this trend, however, can be seen for the shortest molecular weight polycation (40K). For these samples, there appears to be large variability in film break frequency for the thinnest microgel film, which then diminishes as the film becomes thicker with the addition of more layers. Perhaps in these particular thinner samples containing the shorter polycation, more layers are needed to improve the reproducibility properties of the film.

![Figure 5.10. Dependence of polycation molecular weight and number of microgel layers on the “break” frequency in microgel films.](image-url)
To better understand what may be occurring in these multi-layered assemblies, and why the molecular weight of the polycation will affect the strength, it is helpful to consider what occurs when a glassy polymeric material is stretched. Weak areas within the plastically deformation area, or “neck”, start to form microvoids or openings within the material (Figure 5.11b). These voids then coalesce into a propagating crack (Figure 5.11c). Because the materials interrogated in the studies here are actually films and not free standing structures, there are many areas where voids can form and propagate into cracks. This is possibly seen in the preliminary studies shown in Figure 5.11e & f, where the strain induces some opaque regions in the film (perhaps microvoids) that propagate into larger cracks when the strain is doubled. There is a surface energy cost associated with generating the void or new surface, and it is directly proportional to the number of interactions between chains within the polymer.²⁸ A similar relationship can be seen based on the molecular weight of the polycation in the microgel multi-layered film. The films containing the largest molecular weight PDADMAC exhibit the lowest break frequency within the microgel films, and as the molecular weight of the PDADMAC decreases, the break frequency increases. For polycations that have a lower molecular weight, and are therefore shorter, each PDADMAC molecule has less polyvalent interactions with the microgel it has interpenetrated, and also has less polyvalent interactions with the neighboring microgel. Therefore, there are less polyanion-polycation interactions that need to be broken to dissipate the same amount of tensile stress, and therefore is easier to generate surfaces or breaks within the film. The lower number of polycation-polyanion interactions in the film lowers the overall cohesion among constituents in the assembly.
Figure 5.11. Cartoon illustrating defect-induced stretching in materials before (a), and after at strains that cause microvoids (b) followed by crack-propagation (c). Each brightfield optical microscopy to the right shows this possible phenomenon in four-layered microgel films assembled in 400-500K PDADMAC before stretching (d), at 5.5% stretch strain (e) and at 11% stretch strain (f). Scale bar represents 10 µm.

If one were to calculate the average end-to-end distance for each of the PDADMAC chains, assuming a random coil conformation, the polycation’s radius of gyration \( R_g \) would be approximately 32 nm, 18 nm, and 9 nm for 400-500K, 100-200K and 40K PDADMAC, respectively. By assuming that the polymer is in a random coil conformation, which scales by the square root of the number of monomer units, the end-to-end distance, or \( R_g \), can be estimated by multiplying the length of the repeating subunit by the square root of the number of monomer units. The differences between the PDADMAC random coil lengths also appear to scale with the differences seem in the break frequency for the films containing different PDADMAC lengths (approximately 3
µm/cycle, 2 µm/cycle, and 1 µm/cycle for 400-500K, 100-200K and 40K PDADMAC, respectively).

To generate a more accurate relationship between the film break frequency and molecular weight of the PDADMAC, it is important to understand the mechanical forces that have been placed on the sample during stretching. The average tensile stress (σ_{Ave}) on a system equals the applied force (F) divided by the cross-sectional area of the sample (A), or σ_{Ave} = F/A. PDMS is an elastic material, and therefore the amount of applied force can be approximated using Hooke’s law, or F = -kx, where k = the spring constant and x = the displacement. By maintaining a constant stretching displacement, while also assuming the thickness and extent of curing is constant throughout the PDMS used for each sample (which would keep A and k constant), one can assume that the amount of tensile stress applied to every substrate will remain constant. For the studies in this chapter, it is assumed that PDMS is mostly likely dominating the mechanical properties of the microgel-coated PDMS samples because it is expected to have a higher mechanical strength. Therefore, any stress-induced strain on the sample will transmit from the PDMS to the microgel film. PDMS utilized in these studies are 1 mm thick, which have an elastic modulus of approximately 750 kPa. Though the mechanical properties of a dehydrated polyelectrolyte hydrogel film have not been measured, hydrated hydrogel films that have a comparable BIS cross-linking density to the microgels used here have a Young’s moduli on the order of 10’s of kPa. It is unlikely that the elastic moduli of the dehydrated microgel films greatly exceed that of the PDMS. Therefore, because the properties of PDMS dominate the system, it is assumed that the controlled applied tensile stress, which causes the substrate to strain, will transmit the force to the microgel film. Different microgel polyelectrolyte multi-layered films of different thicknesses will have different cross-sectional areas, and therefore experience different amounts of stress. Consequently, it is important to obtain accurate measurements of the microgel film thicknesses. Initially, a masking approach was utilized by placing an object (such as
tape) onto the functionalized PDMS that would remain adhered to the substrate but could also be removed after microgel assembly. Non-residue scotch tape, nail polish, glass, and a smaller piece of functionalized PDMS were all used in an attempt to block formation of the film so that the thickness could be measured. However, the film thickness varied widely (80 nm to 600 nm for one of the films, for example), and the surface of the substrate also appeared non-planar in the AFM Z-sensor images (Z-sensor data is less susceptible to image artifacts than the height data). In the manner that PDMS is functionalized in order to assemble the microgel films, the surface of the PDMS becomes more hydrophilic and therefore can swell in aqueous environments. When one side of the substrate is essentially “pinned” by one of the masks, a swelling instability can occur at the interface and consequently could be contributing to the highly variable film thickness measurements. As can be seen in Figure 5.10, the addition of more microgel layers onto the film appears to have no statistically meaningful effect on the frequency of breaks within the films. However, it is important to at least show that microgels are truly being added to the films with the addition of more layers. Therefore, to eliminate inaccurate film thickness measurements caused by swelling of the substrate, film thicknesses were measured from microgel multi-layers assembled on amine-functionalized glass substrates to interrogate simple film build up. Microgel multi-layered films have been previously measured for film thickness on glass by using a razor blade and scratching away a strip of the film,27 and the same technique was employed here. Such razor blade scratching cannot be performed on the soft PDMS-supported films because the blade cuts the underlying PDMS as well (as mentioned earlier in this chapter). The film thickness data can be seen in Figure 5.12 (a). Beyond a four-layer microgel film containing 400-500K PDADMAC, the film thickness does not appear to increase. For the films containing 100-200K PDADMAC, the film becomes thinner beyond four microgel layers. The 40K PDADMAC film thickness increases slightly only beyond 12 layers. In addition, because microgel films assembled by centrifugation appear to “age” or reorganize while left in
phosphate buffer overnight, as shown in Chapter 3, the affect of aging on film thickness was also interrogated (Figure 5.12 (b)). After aging, the films appear to reorganize, and flatten.

![Figure 5.12](image.png)

Figure 5.12. Film thickness measurements taken before (a) and after “aging” in PBS of 100 mM ionic strength (b).

There are several factors that could be contributing to the film thickness data exhibited in Figure 5.12. One possible reason for the non-step wise addition of microgel layers, when more microgels are centrifuged onto the film, could be attributed the deposition method that was used for their assembly. Perhaps the additional centrifugal force induces a compression on the film, which makes it difficult to de-convolute film buildup from additional microgel layers because of densification of the film. In addition, microgels films appear to rearrange while they are incubated in buffered solution, as evidenced by the changes seen between Figure 5.12 (a) and (b). As was discussed in Chapter 4, microgel monolayers that are deposited via centrifugal force are more densely packed in the lateral direction, and contain smaller footprint areas.²¹ Passively adsorbed microgels are larger in footprint area and less close-packed, and these microgels most likely contain a polymer conformation that is closer to thermodynamic equilibrium. Microgels that are deposited using centrifugal force, however, take on a relatively more condensed polymer chain conformation that is most likely high in energy. Therefore, multi-layered films that are deposited using centrifugal force may be assembled into a
three-dimensional structure that is also not in equilibrium. As the films “ages” in buffer, a reorganization or relaxation may be occurring within the film is solvated as it attempts to dissipate the higher energy non-equilibrium conformation that was induced during the centrifugal deposition process. Another possible explanation for the data shown in Figure 5.12, which could also explain the large differences in film thickness observed for the four-layered films using different molecular weight PDADMACS, could be attributed to the lack of a charge reversal in the film. It is well understood that in order to build polyelectrolyte multi-layered films, after each polyelectrolyte addition, there must be a charge reversal so that a layer of oppositely charged polymer can be added. For films containing the shortest molecular weight PDADMAC (40K), the majority of this polycation may become buried deeper within the microgel relative to the larger molecular weight PDADMACS. Work performed by Bysell et al. showed a similar result when Poly-L-lysine of different molecular weights were allowed to interaction with poly(acrylic acid) microgels. Poly-L-lysine of shorter molecular weight (1-10 kDa) became homogenously confined within the core of the microgels, whereas the larger molecular weight peptides (28 – 170 kDa) were localized to the surface. These results were attributed to slower diffusion of the larger molecular weight peptides in addition to restricted penetration into the microgel by the pore size of the gel network. A similar trend may be present for the microgel films exhibited here, where most of the 40K PDADMAC becomes internalized, and therefore inaccessible to the next layer of anionic microgels. A third possibility, that also leads to lack of charge reversal, could be a complete charge neutralization between anionic microgels and polycation, which does not allow for addition of more layers. The complicated layer formations observed in these results are currently under active investigation.

It was originally believed that the addition of more microgel layers onto the film had no statistically meaningful effect on the frequency of breaks within the films (see Figure 5.10). It is now apparent that it is not clear whether microgels are truly being
added to the film, because the sample thickness does not seem to increase in a step-wise predictable manner with the addition of microgels, as one would expect. Additionally, there appears to be an “aging affect”. It is important to point out that the films assembled on PDMS were not allowed to “age” after assembly like they were on glass because the films already exhibited uniform morphology, which was not the case for the films assembled in the same fashion on glass. The films assembled on glass were much more heterogeneous and “bumpy” in appearance (data no shown). Therefore, the structure of the microgel films assembled on glass versus PDMS is quite different and the true thickness of the microgel films on PDMS most likely cannot be inferred from the film thickness on glass data. Investigations are currently underway to measure the film thickness directly on PDMS using techniques such as ellipsometry, or by measuring film buildup using labeled microgels to better illustrate that additional layers do not affect break frequency. With this data, a more accurate relationship between break frequency of the stretched microgel films and molecular of the PDADMAC can be made.

Stretching was also interrogated on a rigid sphere film containing carboxylated polystyrene microspheres and 400-500K PDADMAC (Figure 5.13. After stretching these films by 11% in length (b) and even by 50% in length (c) & (d), no cracks in the film are visible. The rigid sphere film appears the same as after assembly (a). The inability to observe any cracks in the rigid sphere film may be due to the weak interactions between the microspheres and the underlying substrate compared to the microgel films. Microgels are able to have more points of attachment, or polyvalency, with the underlying amine-functionalized substrate because they can deform and flatten to the surface, whereas a rigid sphere cannot. Therefore, when the PDMS is stretched, the rigid sphere film does not travel with the substrate and has essentially delaminated. Similar to the results found in Figure 5.3, when water is added to the “stretched” polystyrene film, microspheres can be seen floating in the water above the film (Figure
5.11 (e) & (f), and once the film is dried there are areas where significant amounts of particles have desorbed from the film (g) & (h).

**Figure 5.13.** Bright field transmission microscopy of four-layered polystyrene spheres assembled with 400-500K PDADMAC. After assembly (a), stretched by 1 mm (10% of length) (b), stretched by 5 mm (50% of length) (c & d), after water addition (e & f) and dried (g & h). Scale bar represents 80 μm (a – c, e & g) and 20 μm (d, f, h).

To revisit the data shown in Figure 5.4, a comparison of bending damage induced on a four-layered BIS cross-linked pNIPAm-co-AAc microgel film was interrogated. As seen previously, bending of the film while it is present on the outside of the bend results in fractures in random directions (Figure 5.14(a)). However, when the sample is bent with the film present on the inside of the bend, parallel lines form within the microgel film, and little contrast is seen in the brightfield microscopy image (b) compared to what is typically seen for bending of the film when it is present on the outside (a) or when it is stretched (5.9 (a)). As described earlier, when high contrast can be seen in the brightfield transmission microscopy images, as seen in (a), the dark and light regions most likely correspond to high and low microgel density areas. However, the features shown in (b) appear to show lines that are only slightly darker than the rest of the film. AFM was used to better understand the morphological changes in the film during bending when the film is on the inside of the bend (c). By AFM, the weak dark lines correspond to small indents...
in the sample, which may be areas of creasing within the microgel film where microgels have been force to be brought together within close proximity, thus allowing less light to transfer through the film in these areas.

**Figure 5.14.** Brightfield transmission microscopy of damage in a four-layer film using BIS cross-linked pNIPAm-co-AAc and 400-500K PDADMAC. Films were bent with the film on the outside (a) and bent with the film inside (b). Scale bar represents 20 µm. AFM image was included from (b) to show more detailed morphological changes in the film (c).

As discussed earlier in this chapter, it is difficult to directly interrogate the polyelectrolyte multi-layered films as they are healing, because once water is added to the film, it heals within less than a second. It is speculated that one of two rearrangement events are occurring: the film either mends itself back to its original structure once it swells with water addition, or there is extensive lateral microgel reorganization in the areas of damage. To observe which of these possibilities may be occurring, microgels that are fluorescently labeled with fluorescein (AFA) were assembled on functionalized PDMS. These particular microgels had an $R_h = 411$ nm (PD =13%) when dispersed in PBS containing 100 mM ionic strength. In **Figure 5.15** fluorescence microscopy of AFA-microgel films can be seen, and like all the brightfield microscopy images shown earlier in this chapter, the samples are viewed from underneath the film, meaning the light beam travels through the PDMS. The differences between a monolayer (a) and a four-layered film (b) are shown, and more microgels are apparent on the multi-layered film, illustrating microgel buildup as more layers are added. When the film is stretched
(c), or even scratched (d), cracks, that appear bright, can be seen. Scattering of the fluorescent light is most likely occurring at the damage-induced defects in the film. The AFA-microgel multi-layered films are also able to heal when water is added and the sample is dried (e).

**Figure 5.15.** Fluorescence microscopy of four-layered AFA-labeled microgels using 400-500K PDADMAC. View is from underneath the film. Images represent a monolayer (a), four-layer film (b), multi-layer stretched by 1 mm (11% of length) (c), another area where the multi-layer film was scratched (d), multi-layered film healed after water solvation and dried (e). Scale bar represents 20 μm.

To reduce light scattering when the samples were interrogated from underneath the sample, films were stretched and placed upside-down on a glass cloverslip. In **Figure 5.16(a)** & **(c)**, dark cracks in the film are visible, as opposed to bright cracks when the film is interrogated from underneath. These images also further confirm the idea that the damage induced by stretching does cause separations in the film. Healed films are shown in (b) and (d). To interrogate the rearrangement behavior of the fluorescently-labeled microgel films, a focused spot was photobleached in the sample. Therefore, upon a
damaging and healing event, the dark photobleached spot could be relocated and observed for changes in size or relative fluorescence intensity compared to the unphotobleached film. Using the highest magnification objective (100x), a spot was photobleached in the film and interrogated using the 40x objective (Figure 5.17(a)). The film was then stretched by 11% in length (b) and healed by water addition and dried (c). The image in (c) appears very saturated because even at the longest exposure time the film was extremely dim, and therefore the gain was increased to improve visibility. During imaging, extensive photobleaching must have occurred in the area. Four more stretching and healing cycles were performed on the AFA-microgel films to observe whether many damaging events would stimulate more pronounced microgel rearrangement. However, it was difficult to relocate the bleached spot because odd and unexpected defects appeared to be present in the vicinity where the photobleached spot was originally present Figure 5.17(d). The particular sample used for these interrogations was extensively exposed to blue light radiation of the fluorescence microscope while attempting the focused-spot photobleaching experiments. When fluorescein absorbs radiation, it can either transition to the lowest singlet excited state, where upon transition back to the ground state it fluoresces, or fluorescein can transition to the lowest triplet excited state when it absorbs radiation. The triplet excited state can interact with oxygen to form semi-oxidized and semi-reduced radical species that not only cause photobleaching, but have also shown to be able to cause attachment of the fluorescent molecule to proteins via attack by the free radicals. The irreversible damage shown in Figure 5.17(d) may be caused by the polymerization of AFA on the microgels, which would produce a more brittle film that would be unable to heal. Other causes of irreversible damage may be associated with break-down of the underlying PDMS due to prolonged UV exposure or by attack from the photo-generated free radicals. More examinations as to the cause of this irreversible film healing behavior could be the subject of future studies.
Figure 5.16. Fluorescence microscopy of four-layered AFA-labeled microgels using 400-500K PDADMAC. Samples were placed upside-down with the microgel film facing down against the glass slide for imaging. Images represent a multi-layer film that was stretched by 1 mm (11% of length) (a & c), and healed after water solvation and dried (b & d). Scale bar represents 20 µm (a & b) and 8 µm (c & d).

Figure 5.17. Fluorescence microscopy of four-layered AFA-labeled microgels using 400-500K PDADMAC. Samples were placed upside-down for imaging. The 100x objective was used to photobleach a spot, and the film was interrogated using the 40x objective (a). The film was stretched by 1 mm (11% of length) (b), and then healed (c). Stretched and healed five times (d). Scale bar represents 20 µm. Note: the gain was adjusted for (c) and (d) after obtaining (a) and (b).
A new AFA-microgel film was used to re-perform the photobleaching “spot” experiments, and all light exposure was quick so as not to expose the area to extensive amounts of light. In addition, because it took a long time to find the photobleached spot that was focused by the 100x objective, thus prolonging the UV exposure, a spot was alternatively bleached using the 40x objective and interrogated using the 10x objective. The results of this photobleaching experiment are shown in Figure 5.18 under different magnifications. After one stretching (c & h) and healing (d & i) cycle, the photobleached spot continues to appear the same size (approximately 70 μm in diameter) compared to the size of the photobleached spot immediately after photobleaching (b & g). However, the relative fluorescence intensity between the unbleached film and the photobleached spot has changed. Immediately after photobleaching, the bleached spot is very dark while the surrounding film exhibits fluorescence that essentially saturates the detector (b & g). After stretching and then releasing the tensile stress, fluorescence emerges inside the bleached spot, and the film surrounding the spot is no longer saturated (c & h).

Assuming that the rate of photobleaching during imaging of the film is constant between the unbleached film and the photobleached spot, the fluorescence recovery within the photobleached spot may be due to the movement of fluorescent unphotobleached microgels into the bleached spot. The fluorescence within the photobleached spot, relative to the unbleached surroundings, does not appear to change significantly after the stretching damage has healed (d & i). Additionally, after four more additional stretching and healing events (e & j), or five total, the relative intensities appear to remain constant compared to the first stretching damage (c & h). Due to the inability to clearly see the parallel breaks or cracks within the film that is typically seen when the film is stretched, the microscope was switched to transmission mode after the fluorescent microscopy image shown in Figure 5.18(h) was taken. As can be seen in Figure 5.19(a), the parallel separations can be seen in the film and therefore the stretching damage is verified. After obtaining the image seen in Figure 5.18(j) for the film that was damaged and healed five
times total, the microscope was again switched back to transmission mode to confirm that the breaks had been healed (Figure 5.19(b)). These results show that the microgel multilayered films appear to exhibit structural rearrangement in the lateral direction, due to the recovery of fluorescence within the photobleached spot, but the fluorescence recovery appears to occur only after the first damaging event (c & h). Subsequent healing and damaging events maintain the same relative fluorescence intensity ratio between the photobleached spot compared to the unbleached film. Furthermore, the bleached spot does not appear to recover a fluorescence intensity that is similar to that of the surrounding film, perhaps due to the inability for the photobleached bottom film layer, which is covalently attached to the PDMS, to move. These results also suggest that the film rearranges when damaged, but further experiments also suggest that it is possible for the films to rearrange on their own without a damaging event (Figure 5.20). When bleached spots are left in air for 24 hours, there is little change in the relative fluorescence intensity of the bleached spot and the surrounding unbleached film (a & b). However, when the film is incubated in water for only 3 minutes, fluorescence recovery is evident within the bleached spot. These results suggest mobility within the film that is also quite fast. More rigorous and statistical studies are currently underway to better evaluate the mobility of microgel polyelectrolyte multilayered films.
Figure 5.18. Fluorescence microscopy using a new sample of four-layered AFA-labeled microgels assembled with 400-500K PDADMAC. Samples were placed upside-down with the microgel film facing down against the glass slide for imaging. Each image represents a four-layered film after assembly (a & f), a photobleached spot that was burned in 40x objective (b & g), film stretched by 11% of length (c & h), film healed upon water solvation and dried (d & i), film stretched by 11% of length, water healed, and dried five times (e & j). Scale bars represent 80 µm (a – e) and 20 µm (f – j). Note: the “gain” was not changed for any of these images.

Figure 5.19. Brightfield transmission microscopy of stretched (a) and healed (b) AFA-labeled microgel multi-layered (four layers) films. Scale bar represents 20 µm.
It is of interest, for practical purposes, to evaluate whether microgel polyelectrolyte multi-layered films could survive as many as ten damaging and healing events. The repeated durability of these films are shown in Figure 5.21 using the AFA-microgel multi-layered films that were used in the photobleaching experiments above. After performing the five stretching and healing events, an additional five cycles were induced on the film and the presence of microgels was confirmed using AFM.
Figure 5.21. AFM of AFA-conjugated microgels assembled in a four-layer film using 400-500K PDADMAC before (a & c) and after (b & d) stretching by 11% in length and healed 10 times. Scale bars represent 5 µm (a & b) and 2 µm (c & d).

5.4 Conclusion

The work in this chapter clearly shows that microgel polyelectrolyte multi-layers exhibit repeatable self-healing behavior upon multiple mechanical deformations. By assembling the films atop an elastomeric substrate (PDMS), controlled distortion of the film is permitted, thus leading to the direct observation of autonomous film repair. These are the first illustrations of autonomous healing of micron-sized defects in hydrogel films, which is highly relevant in the context of hydrogel-based biomaterials coatings likely to be damaged by routine surgical handling. The self-healing property of the non-fouling microgel film became a critical characteristic for breast implants, as discussed in Appendix A. In addition, LbL assembly has been demonstrated for a vast variety of
materials, thus illustrating the universal utility to the method described here for generating self-healing materials.

The origins of these phenomena likely have their roots in the forces holding the films together at equilibrium. For example, upon mechanical deformation of the elastomeric substrate, the resultant stress is transmitted to the coating. The weak links holding the film together are Coulombic in nature, and it is likely that stress-induced folding or cracking of the film results in some rupture of these polyanion-polycation interactions. The implication that electrostatic bonds are ruptured when the film is damaged is further strengthened by observing what effect changing the molecular weight, or length, of the polycation used as the “glue” layer between microgel layers has on the stretch-induced damage of the film. The larger the PDADMAC molecular weight, the larger the distance between breaks in the film, presumably due to more Coulombic attractions between microgel, PDADMAC, and the nearest neighbor microgel. The lower the PDADMAC molecular weight, the less polycation-polyanion interactions that need to be broken to dissipate the stress. “Tough gels” can also be used as an analogy to the microgel polyelectrolyte films. These gels are said to be tough because they absorb and dissipate the energy from stress-induced strain without breaking, mainly from recoverable elastic deformation. In the microgel polyelectrolyte films, the higher molecular weight polycation can be thought of as having more interactions that can absorb more energy before breaking, and are therefore tougher materials. Rigid carboxylated microspheres cannot exhibit any particle interpenetration by the PDADMAC linear polycation, or particle-particle interpenetration, and therefore have little polyvalency and cohesion between the building blocks. These films exhibit no ability to self-heal.

Since the polycations form both inter- and intra-microgel cross-links, the dissociation of the particles likely leaves an excess positive charge on the particles, in the form of dangling polyelectrolyte chains (net positive surface charge), or bare patches on
the microgels (net negative surface charge). Therefore, the damaged film, which clearly contains regions of both high and low microgel number density, will also be heterogeneous in terms of overall charge. Re-solvation permits higher polymer mobility in the film, which in turn permits a redistribution of microgels to a less energetic state associated with reformation of the polyanion-polycation interactions. In addition, for films that are stretched, the microgels appear to redistribute in the lateral direction just from this simple act. Microgels also show lateral mobility when incubated in aqueous environments simply on their own without a damaging event. These data suggest films that rearrange both when dry and when hydrated, which is indicative of a highly mobile and complex film.

Soft microgel colloidal crystals have also been shown to heal defects, induced by the incorporation of a different sized microgel, within its crystalline lattice. Thus, it may be the case that the self-healing properties of the films described above also have their source in the softness of the interaction potentials between neighboring particles. Though the self-healing capability is not yet completely understood in these two-dimensional microgel structures, the defect-tolerance exhibited within soft colloidal assemblies examined in this other work contributes to the awareness that microgel-based assemblies are intrinsically dynamic and highly complex with respect to their interactions.

5.5 References


CHAPTER 6
EROSION OF SUBSTRATE SUPPORTED DEGRADABLE MICROGELS

6.1 Introduction

Hydrogels have acquired tremendous interest as materials for interfacing with biological environments, as was highlighted in Chapter 1. Microgels and nanogels are relevant for biomedical applications where confined environments must be accessed, such as in targeted drug delivery where extended blood circulation and extravasation into tumors is required. Microgels also benefit from their utility in the fabrication of complex structures via self-assembly, and their high surface-to-volume ratio that maximizes their interaction with the environment. Consequently, microgel-based materials have demonstrated utility in drug delivery, nonfouling films, and biosensors. A large number of the materials used today in biomedical applications ultimately need to be cleared from the body after they have provided their specific function, in order to prevent unwanted side effects or chronic accumulation. Thus, biodegradable constructs are of interest, and achieving a detailed understanding of the morphological changes that occur during erosion is also of particular importance. However, there are no studies that directly monitor degradable hydrogel particles in biologically relevant complex fluids, such as serum, on the single particle scale. In this chapter, atomic force microscopy (AFM) is used to interrogate the morphological and size changes of degradable poly(N-isopropylmethacrylamide-co-acrylic acid) (pNIPMAm-AAc) microgels cross-linked with N,N'-dimethacryloyl hydroxylamine (DMHA) when incubated at physiological temperature (37 ºC) in serum. The cross-linker DMHA undergoes base-catalyzed hydrolysis, and has been used as a cross-linker in macrogels and polymeric particles. At a pH above 5, hydrolysis of DMHA occurs and
it is therefore a suitable cross-linker for rendering microgels erodible under physiological conditions.

There are a variety of techniques by which the erosion of degradable particles can be interrogated, such as by monitoring the loss of fluorescent material,\textsuperscript{17} drug release,\textsuperscript{13,18-20} and molecular weight changes using gel permeation chromatography (GPC).\textsuperscript{18,21} Others have also used optical microscopy,\textsuperscript{13,18-20,22} interrogated the chemical composition after erosion,\textsuperscript{22} or monitored changes in particle light scattering.\textsuperscript{22-23} While these methods can provide important information about degradation kinetics, erosion byproducts, and drug release, oftentimes it is difficult to monitor degradation in complex media, such as serum, due to interference with the analysis. Furthermore, it is important to understand the detailed morphological changes during erosion, which can only be provided by monitoring those changes at the individual particle level, as opposed to relying on ensemble averaged techniques.

AFM possesses several potential advantages over other techniques for monitoring microgel degradation. One obvious advantage is the possibility for direct observation of how a particle changes at particular time points of erosion, and how the changes of a single particle compare to the entire particle population. Furthermore, by using substrate-supported particles, the sample can be incubated in complex media, and then be placed in a more controlled environment for subsequent analysis. AFM, in contrast to other common microscopy techniques such as SEM or TEM, can be trivially performed in both dry and liquid environments, which is exceptionally useful in the interrogation of changes in microgel swelling during erosion.
6.2 Experimental

6.2.1 Materials

All reagents used in this chapter but not listed here are described in Section 3.2.1. Methacryloyl chloride (Sigma Aldrich), pyridine (Fischer), chloroform (BDH), hydrochloric acid (EMD Chemicals Inc.) were all used as received. The monomer N-isopropylmethacrylamide (NIPMAm) was recrystallized from hexanes (J.T. Baker) and dried under vacuum prior to use. Glacial acetic acid (Fischer Scientific), formic acid (EMD Chemicals Inc.), potassium chloride (J.T. Baker), and sodium azide (Sigma Aldrich), were all used as received. Fetal bovine serum was purchased from Atlanta Biologicals, kept frozen until ready for use, and then aliquots were thawed at room temperature before use.

6.2.2 DMHA Synthesis and Characterization

$N,O$-Dimethacryloyl hydroxylamine (DMHA) was synthesized as described previously.\textsuperscript{10} Briefly, 10.1019 g of hydroxylamine hydrochloride (0.145 mol) was placed in a 500 mL round bottom flask and purged with N\textsubscript{2} gas for approximately 20 min to remove moisture. Pyridine, 50 mL, was added by syringe and stirred with the hydroxylamine hydrochloride salt until completely dissolved. Methacryloyl chloride, 23.52 mL (0.243 mol) was slowly added dropwise while maintaining the solution under nitrogen and in an ice bath. The temperature of the reaction mixture was kept below 30 °C, and 10 mL more pyridine was added to the solution after all the methacryloyl chloride was added. The reaction was then stirred for an additional 4.5 hours. Conversion of methacryloyl chloride to product was monitored by thin layer chromatography using a chloroform mobile phase and iodine stain. After complete conversion, 100 mL of chloroform was added and the solution turned a transparent yellow-brown color. Afterwards 21 mL of hydrochloric acid (15 M) was slowly added.
dropwise while the solution was maintained in an ice bath. Vapor formed and the mixture turned turbid. The solution was then poured into a separatory funnel and 100 mL of deionized water was added. The solution was washed with 100 mL portions of deionized water until the aqueous layer became clear (four washes). The organic layer was then dried over magnesium sulfate and filtered. Chloroform was removed by rotary evaporation and then the yellow/brown translucent oil product was dried overnight on a Schlenk line. The product was an off-white solid after drying (7 g isolated). Experimental yield was approximately 34%. The chemical composition of DMHA was determined using a Varian Unity 300 MHz NMR spectrometer and a Perkin Elmer Series II 2400 CHNS/O Analyzer. Chemical composition of the cross-linker N,O-Dimethylacryloyl hydroxylamine (DMHA): $^1$H-NMR (300 MHz, D$_6$MSO, 25 °C): $\delta$ 1.86 [s, 3H, CH$_2$=(CH$_3$)C-CO-NH-CO-C(CH$_3$)=CH$_2$], 1.93 [s, 3H, CH$_2$=(CH$_3$)C-CO-NH-O-CO-C(CH$_3$)=CH$_2$], 5.48 [s, 2H, CH$_2$=(CH$_3$)C-CO-NH-O-CO-C(CH$_3$)=CH$_2$], 5.73 [s, 2H, CH$_2$=(CH$_3$)C-CO-NH-O-CO-C(CH$_3$)=CH$_2$], 5.85 [s, 2H, CH$_2$=(CH$_3$)C-CO-NH-O-CO-C(CH$_3$)=CH$_2$], 6.15 [s, 2H, CH$_2$=(CH$_3$)C-CO-NH-O-CO-C(CH$_3$)=CH$_2$], 11.93 [s, 1H, CH$_2$=(CH$_3$)C-CO-NH-O-CO-C(CH$_3$)=CH$_2$]. An impurity of approximately 9% was found using integrated $^1$H-NMR peaks. Elemental Analysis (CHNO): calculated C-56.80%, H-6.55%, N-8.28%, O-28.37%; found (average of two measurements) C-57.12%, H-6.94%, N-8.39%, O-27.56%. The differences observed between theoretical and found elemental components of the compound are also due to the impurity of the sample. The apparent impurities from $^1$H-NMR and Elemental Analysis are attributed to a small amount of residual pyridine and auto-polymerized DMHA that were both difficult to completely remove. However, it is unlikely that these impurities play a significant role in the synthesis or properties of the degradable microgels.
6.2.3 Microgel Synthesis

Microgels were synthesized by free radical precipitation polymerization using a total monomer concentration of 140 mM, in a molar ratio of 96% NIPMAm, 2% DMHA, and 2% AAc. The initiator ammonium persulfate (APS) and surfactant sodium dodecyl sulfate (SDS) were both used at an 8 mM concentration. The cross-linker DMHA was first dissolved in 5 mL of DMSO, while the rest of the reagents were dissolved in 45 mL of deionized water. The water and DMSO solutions were mixed and filtered through Whatman #2 filter paper via a vacuum filtration system. The polymerization was allowed to proceed for approximately 4 hours at 70 °C under a N₂ blanket.

6.2.4 Microgel Characterization

Dynamic light scattering (DLS) was used, as described in more detail in section 4.2.3), to measure the hydrodynamic radius and diffusion coefficient of synthesized particles. Microgel dispersions were prepared in cold (3 °C) 1X PBS and pH 3 formate buffer of the same ionic strength. Light scattering data was collected at 3 °C. Scattering intensity measurements were performed on a steady-state fluorescence spectrophotometer (Photon Technology International), equipped with a Model 814 PMT photon-counting detector. Scattering was monitored at an excitation and emission wavelength of 600 nm and the temperature was increased from 25 °C to 50 °C at a ramp rate of 0.5 °C/min.

6.2.5 Sample Preparation

Glass was cleaned and functionalized in the same fashion described in section 3.2.4. Microgels were dispersed at a concentration of 0.1 mg/mL in high ionic strength (100 mM) pH 5 acetic acid buffer containing 1 ppm sodium azide. While microgels were dispersing prior to film deposition, they were kept on a shaker table under refrigeration to ensure that no premature degradation was occurring. Cleaned, functionalized, and dried glass disks were individually placed at the bottom of 24-well plates and 500 µL of pH 5
high ionic strength acetic acid buffer was immediately added. The glass was allowed to equilibrate for 30 minutes, and the buffer was then replaced with 500 µL of the microgel solution. Films were generated by centrifugation deposition\textsuperscript{24} at 2250 × g for 5 minutes. Afterwards the films were briefly rinsed with DI water and dried under a gentle N\textsubscript{2} stream.

### 6.2.6 Degradation Studies

Degradable microgel films were incubated in one of four different aqueous environments: (1) 1X PBS-supplemented serum at 37 °C, (2) pH 3 buffer at 37 °C, (3) 1X PBS-supplemented serum at room temperature, and (4) pH 3 buffer at room temperature. To make the PBS-supplemented serum, 1X PBS was first prepared by preparing a solution that contained 10 mM dihydrogen phosphate (24 mM ionic strength), 137 mM NaCl, and 2.7 mM KCl in deionized water, followed by titration to a pH of 7.4. Fetal bovine serum was then diluted to a 10% by volume solution with 1X PBS; this solution was used directly for degradation studies. To make the pH 3 buffer, a solution of 10 mM formic acid was prepared, to which NaCl was added to make the ionic strength 24 mM. Then 137 mM NaCl and 2.7 mM KCl were dissolved in the formic acid-NaCl solution and titrated to a pH of 3. Prior to incubation with microgel films, the buffer was diluted to a 10% by volume solution with deionized water to yield a similar salt content to that of the PBS-supplemented serum.

### 6.2.7 Atomic Force Microscopy and Image Analysis

At a specified time, the film was removed from the incubation solution, rinsed with DI water, and dried with a gentle stream of N\textsubscript{2} prior to analysis. Each sample was characterized using an Asylum Research MFP-3D atomic force microscope. See section 3.2.5 for a detailed description of the AFM instrument and specifications for dry imaging. Three separate images were taken for each sample, and the average particle height was
calculated from 10 particles in each image. Particle heights were determined by drawn line profiles using the analysis panel in the MFP3D software. Lines were drawn across the center of the particle, and the height was measured from the center of the particle to the lowest point on the surface background. In-liquid imaging was performed using an Asylum Research iDrive cantilever with an integrated circuit loop (force constant = 0.09 N/m, resonance frequency = 32 kHz in air) positioned on a specially designed holder (purchased from Asylum Research). For more specifics on the iDrive technique, refer to section 1.3.2.1. A drop of 10 mM pH 5 low ionic strength (24 mM) acetic acid buffer was placed on the microgel film, with the iDrive cantilever lowered into the drop, for 30 minutes to equilibrate before proceeding to take the image in liquid.

6.3 Results and Discussion

Microgels composed of pNIPMAm-AAc cross-linked with DMHA are illustrated in Scheme 6.1. This particular chemical composition was chosen to ensure microgel erosion under physiological conditions (37 °C, pH 7.4, in serum), as opposed to other degradable constructs that require acidic excursions from physiologic pH to induce rapid erosion.25-26 The cross-linker DMHA is known to undergo base catalyzed hydrolysis at pH values above 5, thus making it a useful cleavable cross-linker at a physiological pH of 7.4. The co-polymerization of AAc into the network enabled the microgels to be Coulombically attached to an amine-functionalized surface in order to monitor erosion of particles affixed to a substrate. The concept of Coulombically driving anionic microgels to cationic-functionalized surfaces has been demonstrated previously (Chapters 2 – 5 and Appendix A). The small amount of incorporated AAc (2 mol %) contributes only slightly to a pH-dependent swelling (Figure 6.1a). Lastly, the polymer pNIPMAm undergoes a transition from a water-swollen to a deswollen state upon an increase in temperature above ~41 °C. Thus, these particles are solvent swollen at 37 °C. The
thermoreponsive behavior of these microgels can be seen from the temperature dependent turbidity curve in Figure 6.1b.

Scheme 6.1. Erosion of DMHA Cross-Linked Microgels

Figure 6.1 Size distribution of degradable microgel hydrodynamic radii ($R_h$) determined by DLS (cumulants fit) at pH 3 (filled circles) and pH 7.4 (open circles) (a), and temperature-dependent microgel scattering intensity in pH 3 buffer (b).
After deposition, the degradable microgels were incubated either in the PBS-supplemented serum (pH 7.4) or in the control pH 3 buffer solution. These two particular environments were chosen because the purpose of this study was to observe detailed morphological changes in microgel erosion over time under complex physiologically relevant conditions, and compare these results to microgels that are incubated in a control environment where it is known that the microgels do not degrade. It is possible that serum alone contributes to hydrolysis of the cross-linker, and therefore it was not added to the pH 3 buffer. Erosion was monitored over time either at 37 °C or room temperature. AFM scans were taken in both dry and in liquid (pH 5 buffer) environments at specific time points to evaluate the erosion and change in particle swelling with time. Typical particle erosion over time at 37 °C under dehydrated and hydrated conditions is shown in Figure 6.2. When imaged dry before commencing erosion (Figure 6.2a), the substrate-attached microgels appear hemispherical in shape. After 24 h of exposure to serum, the microgels decrease in height due to the loss of polymer (Figure 6.2b), and after 434 h of exposure the microgel appears to have largely dissolved away, with the exception of a region of high polymer density concentrated in the center of the particle (Figure 6.2c). These particles also appear to exhibit a low-density polymer “ghost” radiating from the dense polymer center. This halo of low polymer density is observed surrounding all microgels after extensive erosion (see Figure 6.3), and is approximately the size of the particle’s original footprint area. The presence of a dense, non-degradable core can possibly be attributed to the presence of pNIPMAm self-crosslinking. Gao and Frisken have observed similar internal crosslinking in poly(N-isopropylacrylamide) gel nanospheres, which they attributed to a chain transfer mechanism. These authors observed nanospheres of pNIPAm, rather than just linear polymer, when NIPAm was polymerized with a free radical initiator above the phase transition temperature in the absence of cross-linking monomers. Therefore, self-cross-linking most likely occurred, and is explained by chain transfer of two active hydrogen atoms that could be attacked by
free radicals: the hydrogen on the tertiary carbon of the isopropyl group and the hydrogen on the tertiary carbon on the main chain backbone. This same self-cross-linking could also have occurred for the pNIPMAm microgels discussed here, thus explaining the presence of a dense non-erodible polymer core after extensive erosion.

Figure 6.2. Three-dimensional renderings of AFM images obtained from a single microgel during erosion at 0 h (a, d), 24 h (b, e), and 434 h (c, f). Images were taken under dehydrated (a-c) or hydrated (d-f) conditions.
Figure 6.3. AFM two-dimensional image of an ensemble of eroded microgels, after 434 h in serum at 37 ºC, illustrating the “halo” effect around a dense polymer center for all particles.

When hydrated, microgels swell to more than twice their height compared to their dehydrated height at all time points during erosion (Figure 6.2d-f). The microgel diameter in Figure 6.2d, before erosion has commenced, is consistent with the microgel size when suspended in solution; the hydrodynamic radius \( R_h \) is approximately 133 ± 6 nm (21 ± 3% PD) in pH 5 buffer. Furthermore, as was observed for microgel erosion under dehydrated conditions, an overall decrease in particle height during erosion is also observed for the microgels when imaged in liquid. The aforementioned low polymer density corona around the dense microgel center observed after 434 hours of erosion is also more clearly visible when hydrated (Figure 6.2f). In addition to the observed changes in particle structure, the background glass substrate becomes noticeably covered with material, which is likely polymer that has detached from the microgels during erosion or protein components from the serum. Due to the slight negative charge of the polymer from acrylic acid, the dissociated polymer chains likely do not re-adsorb onto the microgels, but instead adsorb onto the cationic glass background after release from the microgel.
In addition to the inspection of individual particles, the imaging of multiple microgels allows for observations of the heterogeneity of the entire sample. Larger area scans of the microgel monolayers illustrate that the microgels present within a particular film erode at a uniform rate with uniform changes in particle morphology and swelling. Images illustrating the uniformity of the microgel samples can be seen in the Figure 6.4.

**Figure 6.4.** Two-dimensional AFM height images of an ensemble of degradable microgels at time 0 (a, d), 24 h (b, e), and 434 h (c, f) scanned under dehydrated (a-c) and hydrated (d-f) conditions.

The average dehydrated particle heights as a function of time are shown in Figure 6.5. Erosion at room temperature and 37 °C under both eroding (pH 7.4) and non-eroding (pH 3) conditions are compared here. First, these data clearly display a temperature dependence to the erosion, with the rate being accelerated at higher temperatures, which has been observed previously by others.\(^{18,23}\) Degradation appears to level off at ~100 h and 300 h at 37 °C and room temperature, respectively. In pH 3 buffer at room
temperature, the microgel height remains constant over time, but at 37 °C there is a marked increase in particle height at early time points (after 6 h), which continues to a slow and steady increase over time. It is hypothesized that upon incubation at a higher temperature (37 °C) at pH values where the AAc moieties are protonated, there may be some partial desorption of the microgels from the substrate over time. Indeed, microgels incubated under these conditions appear to have a smaller footprint area, which is perhaps indicative of weaker substrate-particle interactions and therefore less substrate-induced deformation (see Figure 6.6). Note that it is unlikely that the pH-dependent erosion exhibited here is affected by the small amount of acrylic acid comonomer. Microgels that do not contain AAc have also been shown to erode at pH values above 5, and do not erode when dispersed in a low pH environment (pH 3). Furthermore, lack of erosion observed for these microgels in pH 3 buffer cannot be attributed to protonated side chains because the decreased microgel hydrodynamic radius, compared to pH 7.4 buffer (Figure 6.1), is not sufficient to prevent erosion completely. The pH-dependent erosion is largely attributed to the pH-dependent hydrolysis of the DMHA cross-linker.10-11
**Figure 6.5.** Average microgel height over time under dry imaging conditions. Data represents microgel erosion in serum at 37 °C (filled red circles) and room temperature (filled blue circles), and in pH 3 control buffer at 37 °C (open red circles) and room temperature (open blue circles). Error bars represent the standard deviation of the average particle height measured over three separately scanned images.

**Figure 6.6.** Two-dimensional AFM height images, scanned under dehydrated conditions, showing time 0 (a), and 481 h in pH 3 at room temperature (b) and 434 h in pH 3 at 37 °C (c).

To observe microgel swelling at different time points during erosion, AFM was performed in liquid using a selection of the same microgel films used for dry measurements (Figure 6.7). In general, there is once again a decrease in the average particle height over time at pH 7.4, and again degradation is accelerated by an increase in temperature. Control samples (pH 3) do not show significant loss of swollen microgel
height over time (see Figure 6.8). It is also clear from these data that the microgel ensemble exhibits swelling behavior in a uniform fashion. Because swelling behavior in hydrogel materials is dictated by the connectivity and composition of the polymeric network, it is expected that during chain scission of the DMHA cross-linker these microgels will display increased swelling behavior over time. Though this phenomenon is often difficult to observe when polymer mass loss is occurring simultaneously, at early time points during erosion excess swelling can be observed. For example, after 6 hours of erosion at room temperature, there is an approximately 14% height decrease when imaging is performed under dehydrated conditions. However, when the microgels are interrogated following swelling, the height of the partially eroded microgels actually increases slightly relative to the non-degraded particles. This is clearly illustrated by the data shown in Figure 6.9 where mass loss from the microgels is clearly observable in the dry images, but upon swelling the particles appear taller. These data highlight a critical issue in the measurement of erosion via ensemble-averaged techniques. For example, if particle size in the swollen state were used as the main indicator of particle erosion (e.g. by dynamic light scattering measurements), the loss of particle mass would appear to be balanced by cross-link scission and concomitant particle swelling at early erosion timepoints. In other words, the microgel size would appear to stay constant or possibly even increase as erosion proceeded, with large decreases in size only being observable late in the erosion process. Obviously, size alone is a poor metric by which to judge the rate of microgel erosion. Additional difficulties would also arise in the data interpretation when one considers the decrease in scattering intensity that would accompany particle erosion, making the determination of particle size significantly less reliable as the erosion proceeds. Furthermore, the study of microgel erosion by dynamic light scattering, turbidity, multiangle static light scattering, or fluorescence would certainly be difficult to perform in the presence of solvated linear polymer that had sloughed off the remaining microgels, as it would present a significant background signal against which the intact
microgel signal would be difficult to measure. Such background issues are even more problematic in complex media such as serum. Finally, serum components might even cause aggregation of the erosion products or remnant microgels, making analysis even more difficult. Together, these issues illustrate the numerous problems associated with microgel erosion analysis via ensemble-averaged techniques performed on microgel dispersions, and highlight the utility of probe microscopy as an alternative characterization tool.

Figure 6.7. AFM height line profiles taken from an ensemble of hydrated microgels at different time points in serum at (a) 37 °C and (b) room temperature.
Figure 6.8. AFM height line profiles taken from an ensemble of hydrated microgels at different time points in pH 3 control buffer at 37 ºC (a) and room temperature (b).

Figure 6.9. Two-dimensional height images of microgel erosion at room temperature imaged under dry conditions (a, b) and in liquid (c, d). Time points of time 0 (a, c) and 6 hours in serum (b, d) are shown. Scale bar represents 250 nm.
6.4 Conclusion

Understanding the fate of degradable microgels under physiological conditions is potentially important for their application to various biomedical problems. The erosion process involves loss of polymer, changes in particle swelling, and decreased colloidal stability, with complex environments such as serum adding to these complications. Current techniques used to monitor microgel erosion suffer from these complications and, in addition, only reveal ensemble-averaged information about the sample. In this chapter, atomic force microscopy was used to interrogate microgels during erosion in physiologically relevant complex media (serum) on a single particle scale, while simultaneously showing that single-particle changes are indicative of the sample as a whole. Additionally, the ability to interrogate microgel erosion over time under both dehydrated and hydrated conditions allowed for direct and coexistent observations of microgel polymer loss and network swelling associated with cross-linker scission. Importantly, the morphological changes of erodible microgels shown here could only be effectively observed using single particle analysis.

6.5 References


CHAPTER 7
OUTLOOK AND FUTURE DIRECTIONS

7.1 Discussion

It is desirable to be able to control properties of a material’s surface, and in this dissertation, studies were aimed at using microgels for film assembly while also observing and understanding their behavior at an interface. These studies stand on their own as a contribution to the field, however, there still remains several avenues of exploration that one could pursue. In particular, a better understanding of the microgel polyelectrolyte multi-layer assembly, as described in the previous chapters, is needed. In addition, more studies that interrogate the damaging and healing behavior of the microgel polyelectrolyte films, along with film mobility, will lead to more knowledge concerning these interesting materials.

It is unambiguous that the non-fouling microgel multi-layered films are sufficient at reducing macrophage adhesion (Chapter 3). However, the use of centrifugal deposition in making these multi-layered assemblies is still not well understood. Chapter 4 clearly illustrates that this assembly technique, compared to passive adsorption, leads to films containing microgels of smaller footprint size and microgels that are more closely packed. This most likely has an impact on the assembly of the multi-layered film as more and more microgel layers are centrifuged onto the film. It certainly appears to be the case that microgels “age” in solution after using centrifugal deposition (see Figure 3.8), and are perhaps relaxing to reach an assembly that is at an energy minimum. This “energy minimum” is essentially reached when microgels are deposited using a more passive adsorption technique, which should serve as a comparison. In addition, the film thickness measurements seen in Figure 5.12 further illustrate the need to understand why the films do not increase in height in a step-wise
fashion, which we have seen previously using spin-coating.\textsuperscript{1} To interrogate whether the films are being compressed could be accomplished using labeled microgels and monitoring the increase (or lack of change) in microgel density at the surface. Similar fundamental studies could also be performed using Quartz Crystal Impedance to measure the addition of mass, which has been performed previously on spin-coated microgel polyelectrolyte films.\textsuperscript{2} This will give insight as to whether films are actually acquiring more microgels. It is advisable to use passive deposition or spin-coating for future studies involving films until there is a better understanding of how centrifugal deposition effects multi-layer assembly. At the very least, others should be aware of the aging effect.

The work done by Serpe et al.\textsuperscript{2-3} and Sorrel et al.\textsuperscript{1} on the assembly of multi-layered films using anionic microgels and linear polycationic polymer led to the understanding that the polycationic polymer interpenetrated and condensed the microgels. While this may certainly be the case for the multi-layered assemblies discussed in Chapters 3-5, it is still not well understood how this penetration compares when polycations of different molecular weight or different species of polycation (PAH versus PDADMAC) are used. It may be the case that the shortest PDADMAC interpenetrates more deeply, or even to the same extent as the other PDAMAC lengths, but because it is shorter it has less chains extending from the microgel that make it difficult for the next microgel layer to be added. These are assembly characteristics that are still unknown that could be inferred from film thickness or microgel film density data. However, the film thickness data exhibited in Chapter 5 may be convoluted with centrifugal deposition effects, such as compression or higher energy polymer conformations, that make it difficult to truly measure film buildup. It is imperative that one acquires a better understanding of what effect changing the polycation Mw has on film assembly in terms of film buildup, and may be worth repeating using, once again, a more passive deposition (or spin-coating) technique. In addition, by better characterizing the films when the
polycation building block is altered, one can better relate the “break frequency”
dependence on PDADMAC Mw seen in Chapter 5. While the preliminary studies in
Chapter 5 show that break frequency appears to have no dependence on the number of
film layers, it is still not clear whether layers are added at all. It is important to
understand the film thickness of these samples, which cannot necessarily be inferred from
the films assembled on glass, in order to know the amount of stress that was applied to
the film. Other similar fundamental studies should also be geared towards understanding
what affect the microgel particle size, acid content, and stiffness (or cross-linking
density) have on the film assembly and damaging and healing behavior. Studies on
changing the strain amount and rate are also of fundamental interest.

The idea that polyelectrolyte microgel multi-layers are self-healing and also
mobile is incredibly interesting. However, it is still not well understood and there are a
multitude of experiments that one could, and should, perform to better reach an
understanding of the film’s behavior. To start, it is still questionable that the dry damage
exhibited within the film is a result of broken interactions (Coulombic attractions). The
damage most certainly only occurs when the microgel film is present, and therefore is not
an event associated with the PDMS or silicone substrate. If the damage is not a result of
“breaks” or “cracks” within the microgel film, the only explanation would be wrinkling.
There have been numerous studies on the buckling or wrinkling instabilities exhibited by
films assembled on compliant (elastomeric) substrates. However, the films are
typically 1000 fold higher in elastic modulus than the underlying substrate. While the
stiffness of the dehydrated microgel multi-layered assemblies is unknown, it could
possibly be much stiffer than the underlying PDMS substrate (1mm thick PDMS has an
elastic modulus of only 750 KPa). However, if buckling was truly occurring, it is
actually occurring in the direction orthogonal to the applied strain. When stiff films are
assembled on soft supports, and a tensile strain is applied to the sample, a buckling
instability results in wrinkling that is parallel to the direction of the applied strain.
(Scheme 7.1 (a)) because it is compressing in the orthogonal direction. In addition, when this strain is released, the wrinkling vanishes. Studies involving wrinkling instabilities always obtain wrinkling measurements when the samples are under constant strain. In the case of the microgel films (Scheme 7.1 (b)), when the tensile strain is released, parallel line defects appear in the direction orthogonal to the applied strain, and these defects remain present. If the features are wrinkles, then the exhibited wrinkles are actually indicative of a compressive strain in the orthogonal direction to the wrinkling (or same direction as the stretch) upon relaxation. Perhaps upon stretching the film layers slide past each other to generate a wider film that cause a compressive strain upon relaxation. However, one would expect the films to exhibit dramatic changes in the photobleached spot experiments, such as spot deformation, if this were the case. In Chapter 5, though there appears to be some fluorescence recovery within a photobleached spot relative to the surrounding unbleached film, the actual photobleached spot itself does not change shape or size after several stretching and healing cycles. Future studies should be aimed at observing whether the defects in the microgel film truly are “breaks” or some unexpected form of film instability and wrinkling. Using AFM techniques such as Kelvin Probe Force Microscopy could also provide insight as to the distribution of surface potential within a damaged dehydrated film, and possibly illustrate that Coulombic interactions have been disrupted during damaging.
Scheme 7.1. Direction of wrinkling due to buckling instability of a bilayer film containing two materials of different mechanical strength (a) and direction of film features in the microgel film from stretching (b) when stretched in the same direction as (a).

It is also worth noting that it is still unknown what exactly occurs during the deformation process (question mark in Scheme 7.1 (b)). An experimental setup that can apply constant strain while also visualizing the film is needed. This would also be extremely useful to directly visualize how the films behave when damaged under hydrated conditions, because currently the healing has been too fast to be able to effectively observe damage in liquid when the strain is not constantly applied (such as poking and letting go to visualize the area). The Coulombic attraction between polyanions and polycations should be weaker when the film is hydrated, as opposed to dehydrated, and therefore the breaks may occur more often when a wet film is stretched. Investigating damage while the films are in liquid (and in different buffers and salt concentrations) could certainly provide some insight to the dominant interactions within the microgel film.

In addition, controlled and statistically meaningful experiments that illustrate that the films are mobile by simple incubation in aqueous solvent, without a damaging event, should be explored. Fluorescence recovery after photobleaching (FRAP) could be used
to observe changes in the photobleached spot of fluorescently labeled microgel films. Alternatively, a mixture of fluorescently-labeled microgels and non-fluorescently labeled microgels could be assembled, and single particles could be monitored for mobility. These particular experiments are currently underway.

A large number of very interesting materials can be made by exploiting these observed damaging, healing, and mobility behaviors. If the films are truly mobile, perhaps this could be harvested and used for controlled display or localization, where an external triggering event induces a phase separation of labeled microgels. If the defects in the films are truly “breaks” in the film, and the breaks remain in liquid while the film is under strain, then these openings could possibly be filled with other microgels to make super-packed microgel films, or even loaded with other particles or molecules of interest. Mechanical manipulation could also possibly be used as a release mechanism, where the film breaks open to release molecules just by stretching of the film or perhaps even pressing on the back of the film. In the same manner, what if cells that attempt to adhere to the film’s surface cause a mechanical disturbance in the film that leads it to locally crack open and release immunomodulators. In general, however, a better understanding of the overall assembly (effect of centrifugal deposition, changing polycation Mw, etc.) and behavior (understanding what the damage really is, observing damage when hydrated, measuring mobility with and without damage) of the polyelectrolyte microgel assembly building blocks, in conjunction with a better appreciation for the driving mechanisms behind why the microgel assemblies behave the way they do, would be a more practical approach to formulating future experiments.

7.2 References


APPENDIX A

NON-FOULING MICROGEL FILMS ON IMPLANTABLE SUBSTRATES

A.1 Introduction

As discussed in Chapters 2 & 3, non-fouling microgels are effective at reducing protein adsorption and cellular adhesion \textit{in vitro} and reducing the foreign body response associated with implanted biomaterials \textit{in vivo}. Specifically, non-fouling microgel coatings can significantly decrease non-specific protein adsorption, cellular adhesion, and fibrous capsule formation. Up to this point in time, non-fouling microgel films have been tested as coatings on glass\textsuperscript{1,2} or a model biomaterial (PET)\textsuperscript{3,5}. Through recent collaborations with the School of Medicine at Emory University and Georgia Tech Department of Biomedical Engineering, real examples of biomedical implants, such as silicone breast implants and silicon-iridium neural probes, have been provided to evaluate non-fouling microgel coatings in a functional \textit{in vivo} test bed. Both the function and lifetime of these two types of implants have shown to be negatively affected by the foreign body response, and therefore it is presumed that non-fouling microgel coatings can improve their performance \textit{in vivo}. This chapter is dedicated to the assembly of non-fouling microgel multi-layers, in a similar fashion as described in Chapters 3 and 5, to coat two very different implant types (breast implants and neural probes). The ability to coat a variety of implants, varying both in shape, size, and material type, demonstrates the immense utility of using non-fouling microgels as effective barriers between implants and their biological environment for improved functioning and implantation lifetime.

The methods and results discussed here serve as a reference for those that will continue the work so that they can understand what has been previously accomplished.
A.1.1 Breast Implants

Worldwide today, millions of women have breast implants for cosmetic or reconstructive purposes, and these numbers continue to grow because recent studies have confirmed that silicone breast implants do not cause cancer, immune diseases, or other systemic illnesses, as was previously thought. However, breast implants do suffer from local complications and side effects, either over short or longer periods of time. Acute complications include hematoma or infection, while long term complications include implant deflation, wrinkling, rupture, and contracture. In a study conducted by four plastic surgeons from Los Angeles, California, 3495 breast implants were tracked over 25 years, and 514 implants needed to be replaced. The most common cause for replacement was contracture, occurring in 56% of the implant replacements. In addition, breast implant manufacturers report that there is a 10-20% chance that women who receive breast implants will suffer from contracture within 3-5 years. Contracture is the formation of a fibrous capsule that completely surrounds the breast implant, and causes pain, deformation, and hardening of the breast by contracting on the soft implant inside the capsule. Eventually, surgery will be required to remove the capsule (capsulectomy) or to replace the implant completely.

Capsular contracture appears to occur either relatively soon after implantation, understood to be caused by bacterial infection, or later due to the chronic foreign body response. To minimize bacterial contamination, methods such as irrigating the patient’s tissue pocket with antibiotic before implantation, placing the implant underneath the muscle, or using Ioban (antibacterial drapes) are commonly used in practice. While high levels of bacteria have been detected on implants suffering from capsular contracture, relative to non-contracted implants, there are currently no studies that clearly correlate improved sterilization techniques with reduced capsular contracture. For combating chronic foreign body responses, some success has been seen in texturing the surface of the implants, which aims to reduce the ability of adhered myofibroblasts to align with one
another and induce contracture.\textsuperscript{11} Over a period of approximately 7 years, this approach shows reduced incidences of capsular contracture compared to smooth implants. However, after 7 years there is little difference in contracture between the two implant textures, and unfortunately the textured implants increase the incidence of undesirable breast implant waviness or rippling.\textsuperscript{9} Other efforts at reducing chronic inflammatory responses to breast implants have involved the use of ACE (angiostatin-converting-enzyme) inhibitors\textsuperscript{12} or MESNA (sodium 2-mercaptoethane sulfonate),\textsuperscript{13} which lowers the expression of fibrotic mediators or acts as an antioxidant, respectively. Despite these efforts, there is little evidence that the ACE inhibitors or MESNA will continue to reduce capsular contracture over long periods of time after they cease to remain at the surface of the implant. Another approach that has shown great promise at diminishing capsular contracture has been the use of polyurethane foams as breast implant coatings.\textsuperscript{9} However, there have been concerns about carcinogenic degradation products from polyurethane, and these coatings have actually been withdrawn from the U.S market since 1992.\textsuperscript{14} There is a current need for an effective method that safely prevents or reduces the incidence of chronic capsular contracture associated with breast implants.

Non-fouling microgel films exhibit promising characteristics for solving the problem of breast implant capsular contracture. These microgels reduce fibrous capsule thickness \textit{in vivo}.\textsuperscript{5} Whereas this is not a complete elimination of the capsule, a reduction in fibrous capsule thickness may impair the ability of the capsule to contract on breast implants. It is the act of contraction that causes the pain, deformation, and hardening of the breast implant. In addition, the coating process discussed in \textbf{Chapter 3} enables the silicone implant to maintain its original elasticity and flexibility after it has been coated with non-fouling microgels.
A.1.2 Neural Electrodes

The second implant-type discussed in this chapter is a neural electrode (or probe). These micron-sized devices are designed to interface with the nervous system, and are useful for recording electrical signals or providing neural stimulation. Such a device can lead to a better understanding of the nervous system, or serve as prosthetic devices that can improve the life for those that suffer from disorders such as Parkinsons’ disease, deafness, paralysis, blindness, and epilepsy.\(^{15-16}\) However, the long-term functioning of chronically implanted neural probes is compromised by the inflammatory response to an implantation-induced injury to the central nervous system. One result of this inflammatory response is the formation of a glial scar.\(^{17-18}\) The glial scar forms a barrier between the electrode and surrounding neurons. In addition, when a foreign object is inserted into neural tissue, neurons and glial cells (cells that provide support and protection for the brain’s neurons) are killed or injured, and macrophages infiltrate the area of injury from the disrupted blood vessels. Shortly after, astrocytes, macrophages, and oligodendrocyte precursors, which are the three main cell-types present at the injury site, express CSPGs (chondroitin sulfate proteoglycans), which inhibit neural cell regeneration.\(^{19}\) Both glial scar formation and the loss of neural cells surrounding the implanted electrode lead to electrical impedance.\(^{20-21}\) Therefore, to improve the ability of the neural electrodes to perform electrical readings or to send electrical signals, the inflammatory response needs to be modulated to reduce both glial scar formation and neuronal cell death.

Several studies have been conducted in an attempt to lower the electrical impedance associated with neural probes. One such approach is by minimizing trauma to the tissue during implantation by altering the size, shape, geometry, texture, material, and insertion procedures.\(^{17-18,22-26}\) Another method is to improve conductance by depositing metals or conducting polymers to the probe’s surface.\(^{27-28}\) Other approaches have focused on reducing the foreign body response that leads to glial scar formation by
utilizing bioactive coatings or delivering anti-inflammatory drugs. The majority of these methods, however, do not reduce the electrical impedance over long periods of time, or require high complexity by incorporating many of the approaches listed above.

Simple non-fouling microgel coatings have been able to decrease the foreign body response associated with biomaterials in vivo, and therefore it is postulated that these coatings will be able to modulate the foreign body responses in neural tissue as well, thus decreasing the electrical impedance associated with chronically implanted neural probes. In addition, other relatively thick hydrogel-coated electrodes experienced a 20% increase in impedance, but the highly hydrated microgel films exhibited here are much thinner (over 100 fold) and are therefore not anticipated to significantly increase the electrical impedance.

A.2 Experimental

A.2.1 Materials

All reagents used in this chapter but not listed here are described in Section 3.2.1. Sodium azide (Sigma Aldrich) was used as received. Miniature 10 cc and 50 cc silicone gel filled breast implants were manufactured and donated by Mentor Corporation (now owned by Johnson & Johnson). Hydrochloric acid was purchased from EMD and used as received. Silicon-iridium neural probes were purchased from NeuroNexus Technologies (Ann Arbor, MI). Trichloroethane (JT Baker), acetone (Sigma Aldrich), and methanol (Sigma Aldrich) were used as received. The Limulus Amebocyte Lysate (LAL) endotoxin screening kit was purchased from Lonza (Walkersville, MD)

A.2.2 Microgel Synthesis

Synthesis of non-fouling microgels used this chapter are described in Section 3.2.2. Instead of performing syntheses in a 50 mL total volume, syntheses were performed at 100 mL volumes.
A.2.3 Implant Surface Functionalization & Microgel Film Assembly

A.2.3.1 Silicone Breast Implants

The 10 cc miniature implants were cut completely in half and the silicone gel within was scraped out. To completely remove all the gel, the casing was incubated in hexane overnight, which softened the gel for easy removal. The remaining adhered gel was wiped off using a gloved hand. The casing was then cut into 8 small slivers. The bottom part of the casing, which contained a dense ring of silicone elastomer, was avoided. The silicone pieces were functionalized in the same manner as described in section 5.2.3. Microgel monolayers were covalently attached to the amine-functionalized silicone using the same method described in section 3.2.4 with the exception that the microgels were passively adsorbed overnight instead of centrifugally deposited. Four-layer microgel coatings were assembled on the breast implant using the method described in section 3.2.4, and again the microgel layers were not deposited by centrifugation but by passive adsorption overnight. Film coverage and stability was evaluated using atomic force microscopy (AFM). Details on AFM can be found in section 3.2.5.

Fourteen of the larger 50 cc implants were kept intact and functionalized using the same method described for the silicone breast implant pieces with the exception of cutting and incubation in hexane. Large 250 mL jars with plastic-lined lids was used to submerge the implants so that they were not touching the sides of the jars, and all incubation steps were performed on the shaker table. The volume of the solutions used was kept to 100 mL, which was sufficient to submerge the implant without excessive waste of solution. Two smaller implant pieces from the 10 cc implants were functionalized and coated in parallel so they could be interrogated by atomic force microscopy to verify coverage.

In preparation for implantation in swine, all jars and equipment were incubated in a 250 °C oven for 30 minutes, followed by cooling in a laminar flow chemical hood with
the stash closed. The fourteen 50 cc implants were cleaned in 70 % aqueous ethanol for 24 hours after coating with microgels, followed by incubation in Lonza endotoxin-free water for 1 hour to remove the absorbed ethanol. Fourteen control implants were also prepared in the same manner. An LAL endotoxin screening assay was performed to determine if the implants contained endotoxin after the cleaning procedure. Following the procedure outlined by the FDA, implants were washed in 40 mL of endotoxin-free water, and endotoxin levels were below 0.5 EU/mL of entoxin. This is the acceptable endotoxin level specified by the FDA for medical devices.\textsuperscript{34} Furthermore, aliquots from the 40 mL wash from the cleaned implants were spiked with endotoxin to generate a 1 EU/mL solution to verify no inhibition of endotoxin activity. After all implants were cleaned in ethanol, followed by incubation in endotoxin-free water, the water was removed and the samples were sealed in their cleaned glass jars for transport to Emory University. Prior to implantation, implants were incubated in a saline solution containing bacitracin for approximately 5 minutes. Implants were placed between the subcutaneous fat and muscle of seven miniature pigs. Each pig received four implants, two coated implants and two control implants, with two on each side. Swine were euthanized at time points of 1 and 2 months. Explanted implants were evaluated by optical microscopy using a 100x objective (refer to section 5.2.4). Samples from the explanted implants were extracted using gentle pressure and a clean sharp razor blade for analysis by AFM. Details on AFM can be found in section 3.2.5. Tonometry and histology were used to evaluate the extracted capsule (data is not shown in this thesis).

A.2.3.2 Silicon-Iridium Neural Electrodes

The probes used in these studies contained four shanks (prongs) that are approximately 5 mm long, 15 \( \mu \)m thick, and 200 \( \mu \)m wide. Each shank contains imbedded iridium disks (15 \( \mu \)m in diameter) used for sensing electrical signals within neural tissue, and raised iridium channels that carry the signal to the other end of the
probe. The probes used here are “dummy” probes that do not contain any of the electrical circuitry that a real probe would have. Due to their fragile nature, a gentle yet effective cleaning method was needed. The most promising consisted of incubation in the following solvents for 5 minutes each: trichloroethane, acetone, and then methanol. The surface was silanized by incubating the probe in absolute ethanol for 30 minutes, followed by incubation in 1% APTMS by volume in absolute ethanol for 2 hours.

After amine functionalization, microgel monolayers and multi-layered films were assembled on the probe using the same method described above for breast implants. Probes were evaluated for microgel surface coverage by atomic force microscopy. Probes were also sent to National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO) (University of Washington, Seattle, WA) to determine surface atomic composition by X-ray photoelectron spectroscopy (XPS).

A.3 Results & Discussion

Non-fouling microgel films have demonstrated their effectiveness at reducing the foreign body response associated with implanted biomaterials (see section 2.3). In Chapter 3, their assembly into multi-layers and ability to reduce macrophage adhesion to glass has also been demonstrated. In this chapter, non-fouling microgel multi-layered films are assembled on functional examples of medical devices that are made of silicone or silicon by utilizing a similar surface functionalization method to that of glass.

A.3.1 Breast Implants

The breast implants studied here contain a silicone-elastomer shell. Most manufactured implants also contain a shell made of silicone. After cutting the implant into pieces, hexane was used to effectively remove the exposed gel, so that it would not interfere with the coating method. The surface of these silicone casings can be seen in Figure A.1a, and are actually fairly rough in appearance. The hexane-cleaned silicone
pieces were then incubated in a solution of microgels overnight. While it appeared that microgels did interact well with the surface of the silicone in some areas (Figure A.1b), there was patchy coverage in other areas (Figure A.1c). The inability to provide complete and continuous coverage of non-fouling microgels on the breast implant casing will result in an ineffective barrier against the foreign body response. In addition, the microgel-silicone interaction is not covalent. In the past, the Lyon group has demonstrated the ability to attach anionic microgels by Coulombic attraction to the surface of amine-silanized surfaces.\textsuperscript{2,35-38} Fortunately, the surface chemistry of silicone can be made similar to that of glass and then functionalized using common silane chemistry. The most common method for introducing surface hydroxyl groups on the surface of silicone is by exposure to ultraviolet ozone or oxygen plasma.\textsuperscript{39-44} UV ozone treatment and plasma oxidation, however, produce brittle silicate or silica-like surfaces, which are highly undesirable for breast implants. An alternative to UV ozone or oxygen plasma is hydrochloric acid incubation.\textsuperscript{45} By exposing silicone to HCl, the acid cleaves the silicone-oxygen backbone and generates silanol groups as a result.\textsuperscript{46-47} Because silicone is extremely hydrophobic, aqueous solutions are not able to penetrate the bulk of the material. Therefore, the acid-induced cleavage is confined to the surface of the silicone and a stiff oxide layer is not produced, thus allowing the substrate to remain soft and flexible. After incubation with a solution of HCl, the generated surface silanol groups are then functionalized with 3-aminopropyltrimethoxysilane (APTMS), followed by dip-coating in a solution containing non-fouling microgels. The monolayer assembly can be seen in Figure A.2, where microgels can only be observed in the AFM phase trace due to the roughness of the elastomeric breast implant casing (Figure A.2b). The microgel monolayers present in Figure A.1b are more noticeable, or taller, compared to the monolayers in Figure A.2b. This is indicative of a different microgel-silicone interaction, where the microgels are flatter, and possible more intimately associated, with the amine-functionalized silicone. The monolayers present in Figure A.2b were
covalently attached to the surface, and 3 additional microgel layers, with PDADMAC as the cationic “glue” in between microgel layers, were assembled on top. The multi-layered films can be seen in **Figure A.2c & d**, which were found on the surface of the breast implant casing in all places imaged.

![Figure A.1](image1.png)  
**Figure A.1.** AFM height images of hexane-cleaned silicone (a), and a microgel monolayer on cleaned silicone (b & c). Scale bar represents 5 µm.

![Figure A.2](image2.png)  
**Figure A.2.** AFM height image (a) and phase image (b) of a microgel monolayer on functionalized silicone. Two representative height images of four microgel multi-layers (c & d) assembled on top the monolayer shown in (a & b). Scale bar represents 5 µm.

To evaluate the stability of the non-fouling microgel polyelectrolyte multi-layered coatings assembled on functionalized silicone, microgel coated pieces were subjected to environments they would encounter during the preparation and use as a biomedical implant. First, the sample was incubated in 70 % aqueous ethanol for 24 hours, which is a cleaning solution that has been used previously to prepare microgel films for cell culture. The sample was then incubated in 10 % serum supplemented in 1X PBS (phosphate buffered saline) at 37 °C for 24 hours. The intact microgel film can be seen in **Figure A.3a & b**. A smaller-sized scan of a plain non-coated silicone substrate was
included for comparison (Figure A.3c) to show the relative topographical features compared to a smaller-sized scan of microgel-coated silicone (Figure A.3b), indicating that microgels are indeed present in the coated sample.

In addition to chemical stability in ethanol and under physiological conditions, the breast implant coating must be mechanically stable as well. Breast implants are surgically handled when implanted, therefore subject to poking, rubbing, scraping, bending, and rolling. A soft colloidal hydrogel coating, consisting of non-covalently interacting particles, is not expected to withstand such mechanical manipulation. In fact, as can be seen in Figure A.4a, the simple act of flattening the coated silicone casing onto a taped glass slide for imaging seemed to disrupt the microgel multi-layered film in some areas. To interrogate whether these discontinuities within the film would result in delamination of the coating during handling, the film was poked with a gloved hand (Figure A.4b). It appeared as if the breaks in the film were pressed back together by this simple act. The film was further subjected to vigorous rubbing with a gloved hand (Figure A.4c), which regenerated the film discontinuities. In-liquid AFM imaging was attempted to observe whether hydration of the film would allow the microgels to swell and reduce the defects within the film by decreasing the size of the gaps. However, it was difficult to produce a clear scanned image during in liquid AFM imaging, let alone being able to locate the gaps. When the hydrated films were dried and imaged again under dehydrated conditions, the defects were nowhere to be seen (Figure A.4d). The self-healing phenomena observed here are described in detail in Chapter 5, where microgel multi-layered films assembled on soft substrates are able to undergo repeatable damaging events and instantly heal once solvated with water. To further illustrate the resilience of microgel multi-layered films, non-residue adhesive tape was placed on the film in an attempt to pull off significant amounts of microgels. However, after this “tape test”, the microgel film appeared to remain intact (Figure A.4e).
Figure A.3  AFM height images of microgel multi-layers after incubation in 70 % aqueous ethanol for 24 hours, followed by incubation in 10 % serum supplemented in 1X PBS at 37 °C for 24 hours (a & b). Cleaned silicone (c) for comparison. Note that (b) and (c) are on the same z-scale. Scale bars represent 5 µm (a) and 2 µm (b & c).

Figure A.4. AFM height images of microgel multi-layers after taped to slide (a), pressed down upon (b), rubbed vigorously (c), incubation in water while attempting to interrogate the film via in liquid AFM (d), and after the “tape test” (e). Scale bar represents 5 µm.

In this chapter, it has been shown that non-fouling microgels can be assembled on silicone breast implant casings in a polyelectrolyte multi-layer fashion. In addition, these microgel coatings are able to withstand cleaning in ethanol, incubation in biological media, and mechanical manipulation. Furthermore, it has been shown previously (Chapter 3) that such microgel assemblies can effectively minimize macrophage adhesion. In light of these findings, small 50 cc silicone breast implants were coated to be tested in a swine model. A swine model was chosen because the skin and subcuticular tissue of pigs most appropriately mimic the human breast tissue compared to other mammals, and a miniature pig model has been established previously as an appropriate model for interrogating the formation of the fibrotic capsule. Dr. Hunter Moyer, a practicing resident from the Division of Plastic Surgery at Emory University, implanted the breast implants into swine and removed them after 1 and 2 months. Optical
microscopy of the one month explantations can be seen in Figure A.5. The coated implants appear to have “cracks” that the uncoated explant does not possess. These “crack” patterns are also apparent in the coated implant piece that was never implanted. The cracking patterns also appear in Figure A.4 for the microgel-coated silicone casing that was mechanically manipulated. Furthermore, it is known that these deformations or cracking events are characteristic of the microgel multi-layer assemblies on soft substrates (see Chapter 5). In addition, AFM of the 1 month explants can be seen in Figure A.6. The spherical objects on the explanted microgel coated implant look similar to the coated implant sample that was never implanted. A smaller z-scale was used for the smaller scan-sized image of the uncoated implant (Figure A.6e) to illustrate that the spherical objects appear much smaller, and are distinguishable from the microgel coated samples. Therefore, it appears from both the optical microscopy and AFM interrogations that the microgel film remained intact and present on the breast implant during the one-month implantation period. However, additional studies or more samples are necessary to strengthen this argument. Implants were also interrogated at the 2 month time period. There were no distinguishable features between the microgel coated and not coated implants by transmission microscopy (data not shown). AFM of the 2 month explantations are shown in Figure A.7. There were two distinct regions seen by eye without magnification on the implants: an opaque region and a somewhat clear region. These regions were interrogated separately by AFM. The clear region of the coated implant and uncoated implant actually appear very similar (a & c respectively) by AFM, which may indicate that significant microgel desorption occurred after two months of implantation. The opaque regions also appear somewhat similar for the coated and uncoated implants (b & d respectively), and it is difficult at this time to be confident in what these features represent. The “crack” seen in Figure A.7b may be similar to the cracks seen in the 1 month samples, but these same features are visible in the non coated sample as well. The raised regions could be anything from a biofilm to a weak spot in
the silicone elastomeric shell of the implant. A breast implant that was taken straight out of the manufacturer’s box, that was also implanted for the two month period, was included for comparison (Figure A.7c) to see if our sterilization procedure (ethanol incubation) may be inducing damage to the implant. While that possibility is not clear at this time, it does appear that the implant not subjected to ethanol incubation for 24 hours has an overall smoother surface (with the exception of a few spots that may be adsorbed biological material) for the entire implant. Further studies are warranted to better understand how the breast implants are affected by ethanol incubation and microgel-coating procedure.

![Figure A.5.](image)

**Figure A.5.** Transmission optical microscopy of microgel coated breast implants (a & d) and uncoated breast implants (b & e) that were explanted from swine after 1 month of implantation. A coated implant piece that was never implanted was included for comparison (c & f). Scale bars represent 10 µm (a-c) and 5 µm (d-f).
Figure A.6. AFM height images of an extracted microgel coated breast implant sample (a & d) and extracted uncoated implant sample (b & e) after 1 month of implantation. A microgel coated non-implanted piece was included for comparison (c & f). Note that a-c and d & f are on the same z-scale.

Figure A.7. AFM height images of 2 month explantations: microgel-coated implant (a & b), uncoated implant (c & d), and uncoated implant that was not subject to the same sterilization procedure and taken straight out of the box from the manufacturer (e). Scale bar represents 5 µm.

In addition to the topographical interrogations of the film after implantation, laboratory culture results (performed at Emory University) determined that “scant growth” of bacteria was detected on all surfaces. Explanted microgel coated implants were also bright yellow, compared to uncoated implants that varied from being slightly yellow to clear. This may be indicative of contamination that was not effectively removed during the sterilization procedure or could not be detected by endotoxin screening (which only detects for gram negative bacteria). Indeed, microbial biofilms
can appear yellow. The microgel coated implants may have provided a surface in which the bacteria could proliferate at a higher rate than on the uncoated implant, thus resulting in a relatively brighter yellow color. Another study has also shown a “yellow stained” poly(ethylene glycol)-serum albumin hydrogel that was implanted for 7 days, but there was no offered rationale for this observation. Furthermore, ethanol induces swelling of the breast implant and may lead to silicone gel bleeding. Both the silanization and sterilization methods used involve incubating the implant in ethanol, and after the breast implant has been transferred from ethanol to an aqueous solution, the implant becomes turbid in appearance. Upon closer inspection using optical microscopy to image beyond the silicone casing and inside the breast implant, large spherical objects are visible and may be air bubbles. These bubbles could be due to a reorganization of the gel after it has swelled in ethanol, or areas where gel has bled out from within the casing. Current efforts are focused on eliminating the use of ethanol in the method of silanization (by using vapor silanization as an alternative) and also as the cleaning procedure. An effective sterilization method is also needed to eliminate bacterial growth, such as the commercial solution Alcide (active ingredient of sodium hypochlorite) or Sporelenz (active ingredient of hydrogen peroxide).

Mechanical compression testing and histology data of the fibrous capsule from the two month implants revealed a thicker more contracted capsule on the coated implant when compared to the controls (data not shown). However, because the microgel coated implants also appear to be harboring bacteria, which have been shown to be associated with contracture as mentioned in section A.1.1, it is possible that these capsule results are a consequence of a proliferating biofilm and not directly related to the microgel film. In addition, the uncoated breast implants that were taken straight out of the manufacturer’s packaging had a thinner capsule compared to the uncoated implant that had been sterilized using ethanol incubation. Therefore, it is possible that the foreign body response may have been heightened by ethanol swelling induced gel leakage.
Before performing these in vivo studies for a second time, both a different surface functionalization technique that does not involve the use of ethanol, and a more effective sterilization procedure (not involving organic solvents) that can also be evaluated for effectiveness at minimizing levels of various types of bacteria are needed.

### A.3.2 Neural Electrodes

The neural probes studied here were composed mainly of silicon, which can also conveniently be functionalized using silanization chemistry. However, several attempts at functionalizing the probe resulted in inconsistent and patchy microgel coverage. It is generally understood that in order to perform silanization chemistry effectively, the surface of the substrate needs to be impeccably clean. Therefore it is possible that the probes interrogated here may possess contaminants on the surface that are contributing to the inconsistent microgel coverage, and cleaning techniques such as oxygen plasma are insufficient at ridding the surface of these contaminants. Indeed, the surface of the probe appeared “dirty” by AFM. In Figure A.8, unknown materials are clearly visible, which may come from organic solvents or other substances, such as photoresists, that are used during the micro-fabrication process of these probes. Further analysis of the probe’s surface by X-ray photoelectron spectroscopy (XPS) revealed the atomic species present before oxygen plasma exposure, after plasma oxidation, and after silanization with APTMS (Table A.1). A high carbon content is evident on the surface of the probe, along with a small amount of nitrogen and inorganic elements, when it is taken directly out of the manufacturer’s box. Plasma cleaning appears to remove all inorganic material from the probe’s surface, while also reducing the nitrogen content, but the amount of carbon increases. Functionalization with APTMS regenerates the nitrogen content, and increases the carbon content, which is expected. However, there should not be any carbon residues on the probe after cleaning with plasma oxidation. Therefore, a more effective cleaning procedure is needed to remove these organics from the probe’s surface.
Figure A.8. AFM height profile scan of a neural probe taken straight from the manufacturer’s box. Scale bar represents 5 µm.

Table A.1. XPS data showing atomic compositions from the neural probes surface.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carbon</th>
<th>Calcium</th>
<th>Iridium</th>
<th>Nitrogen</th>
<th>Sodium</th>
<th>Oxygen</th>
<th>Silicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Out of Box</td>
<td>39</td>
<td>2.1</td>
<td>0.5</td>
<td>3.6</td>
<td>0.7</td>
<td>40.1</td>
<td>14</td>
</tr>
<tr>
<td>Oxygen Plasma Cleaned</td>
<td>49.6</td>
<td>0</td>
<td>0.2</td>
<td>0.3</td>
<td>0</td>
<td>39.6</td>
<td>10.3</td>
</tr>
<tr>
<td>APTMS functionalized</td>
<td>58.2</td>
<td>0</td>
<td>0.4</td>
<td>4.7</td>
<td>0</td>
<td>28.4</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Recent results obtained from Stacie Gutowski of the Andrés García lab at Georgia Tech suggest that by incubating the probe can be sufficiently cleaned in a solution of trichloroethane (TCE), followed by acetone, and then deionized water. At this point in time, reproducible results have illustrated that probes that have been cleaned by this procedure can be completely and effectively coated with non-fouling microgel multilayers (see Figure A.9).
A.4 Conclusions and Future Directions

A.4.1 Breast Implants

In this appendix, non-fouling microgels were assembled in a polyelectrolyte multi-layered fashion on silicone breast implants. These microgel assemblies are both chemically and mechanically robust, in addition to previously showing reduced unwanted cellular adhesion, and therefore are practical and useful as a breast implant coating for minimizing chronic capsular contracture. In addition, the coatings appear to remain intact after 1 month of implantation in swine. However, in discussions with the reconstructive surgeons at Emory University in regards to laboratory culture results (mentioned earlier in section A.3.1), bacterial contamination was prevalent throughout the tested samples, thus making it difficult to draw conclusions about the fibrous capsule histology results and mechanical compression data related to the microgel coating. An aqueous sterilization method, which is effective at thoroughly eradicating microorganisms on the implants while not inducing implant swelling, needs to be
employed and tested for efficacy. In addition, ethanol was used to functionalize the implant’s surface, which may have caused silicone gel to leak from the implant. New efforts are also focused on functionalizing the implant surface using vapor phase silanization so that the use of ethanol can be avoided. There is also interest in imbedding antibacterial agents, such as silver ions, to improve the antibacterial properties of the non-fouling microgel coatings.

A.4.2 Neural Electrodes

In addition to breast implants, neural probes could also benefit from non-fouling microgel coatings that would minimize the formation of a glial scar and neuronal cell death in the vicinity of the implantation site, which would in turn reduce the impedance of the electrical signals that the probe is attempting to collect from the surrounding nervous tissue. Currently the probes appear to be coated well, and their stability as they are inserted into brain tissue is the next object of study. The electrical impedance must also be evaluated to ensure that the microgel coating itself does not deter the electrical signals being received by the probe. Future work could focus on the release of immunomodulators to modulate the host’s response to implanted neural electrodes.

A.5 References


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VITA

Antoinette Bonhivert South (maiden name Antoinette Christine Bonhivert) was born on March 2\textsuperscript{nd}, 1983 in Caracas, Venezuela. She attended Robinson Secondary High School in Fairfax VA, where she received an International Baccalaureate (IB) diploma in 2001. She then went on to obtain her B.S. in Chemistry from James Madison University in Harrisonburg, Va (2005) before pursuing a doctoral degree in Chemistry from Georgia Institute of Technology.