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Preface

The Journal for Undergraduate Research in Bioengineering (JURIBE) has been a successful vehicle for undergraduates to publish their research results. This 2002 publication reflects a continued partnership between two National Science Foundation Engineering Research Centers, the University of Washington Engineered Biomaterials (UWEB) Research Center and the Georgia Tech/Emory Center (GTEC) for the Engineering of Living Tissues.

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The discussion should interpret and discuss the importance of the findings. It should also compare the findings to previous results in the field and to predictions by theoretical models, as appropriate.

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Acknowledgements of the author’s advisor and institution are encouraged. Funding information for grant-supported programs (i.e. National Science Foundation, National Institutes of Health) should be listed as well.

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I. INTRODUCTION

1. Engineered Surfaces in Biology and Medicine

With the recent advances in biomedical sciences, the need to control surface chemistry has become apparent. Biomedical surfaces need to maintain their integrity when placed in the body’s environment and trigger natural healing mechanisms while remaining resistant to random protein adsorption and fouling. To elicit these desired responses, biomaterial surfaces comprised of detailed, complex chemistries are required. To this end it must be understood how controlling the surface chemistry of a material can effect its interaction with cells and proteins [1,3].

1.2 Self-Assembled Monolayers: Model Organic Surfaces

Self-assembled monolayers (SAMs) provide well-defined structures and chemistries that can be easily fabricated and systematically varied [1,3]. SAMs are constructed by spontaneous adsorption of thiols [SH(C\textsubscript{H}\textsubscript{2n})\textsubscript{R}x] onto a metal (usually gold, copper, or silver) surface [3]. The composition of the final layer formed will depend on the thiols used in the assembly solution and the interfacial properties of SAMs can be controlled by changing the functional group “R” [8]. This unique feature makes SAMs ideal surfaces for studying many biological reactions such as protein adsorption.

1.3 Poly(Ethylene Glycol) Self-Assembled Monolayers

One area of interest in the biomaterials field is the development of non-fouling surfaces. Understanding the mechanism of protein adsorption at surfaces is an important aspect in creating successful biomaterials. One system that has been recognized by many as a controllable, protein-resistant surface is poly(ethylene glycol) (PEG) SAMs [5,6,8]. The debate over the mechanism of PEG’s resistance to protein is ongoing, however.

Jeon et al. proposed that the resistance of PEG to protein adsorption is a steric repulsion effect in which the polymer chains prevent the protein from reaching the surface to adsorb [6]. More recently, however, Harder et al. proposed that the order of PEG surfaces affects their non-fouling properties [5]. To date, no consistent method has been employed to characterize PEG SAM surfaces that would allow this recent hypothesis to be fully tested.

1.3 Research Objective

Previous work at the University of Washington has shown that pure monolayers of HS(CH\textsubscript{2})\textsubscript{11}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{4}OH (PEG4) form disordered surfaces. Based on the data collected, the following factors were found to promote order in mixed PEG monolayers: longer CH\textsubscript{3} terminated thiols, approximately a 50/50 PEG4/CH\textsubscript{3} thiol ratio in the monolayer, higher assembly temperatures (60 - 65°C), and shorter assembly time (1 hour rather than 2 days). The correct combination of these factors needed to create truly ordered mixed PEG4 self-assembled monolayers is still under investigation.
gold via electron beam evaporation. Three thiols were used in the preparation of SAMs: SH(CH₃)₁₁CH₃ (C₁₂; 1-dodecanethiol; Aldrich Chemical Company, 98% pure), SH(CH₂)₁₇CH₃ (C₁₈; 1-octadecanethiol; Aldrich Chemical Company, 98% pure), and SH(CH₂)₁₁(OCH₂CH₂)₄OH (PEG₄; synthesized in house).

All surfaces were assembled from 1 mM solutions in pure ethanol (EtOH; Ricca Chemical Company, ACS Reagent Grade). Mixed SAMs were prepared using v/v assembly solutions. When needed, thiol solutions were sonicated for approximately 5 min. to ensure complete mixture of thiols and solvent. Sample Sets 1 and 2 were assembled in 20 mL capped glass vials and Sets 3-6 were assembled in 14 mL capped polystyrene test tubes. All samples were backfilled with and sealed under gaseous nitrogen.

After assembly, all samples were rinsed for 10 sec. with pure EtOH from a solvent rinse bottle. Any samples containing PEG₄ thiol were then sonicated in fresh EtOH for 2 min. and re-rinsed to remove any physisorbed thiols. Samples were then dried with nitrogen gas, placed in polystyrene petri dishes, backfilled with and doubly sealed under gaseous nitrogen. For specific assembly information used in

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Assembly protocol used in preparing Sample Set #1</th>
</tr>
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<tr>
<td># of Samples</td>
<td>% PEG₄ (v/v)</td>
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<td>100%</td>
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<td>0%</td>
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<tr>
<td>2</td>
<td>90%</td>
</tr>
<tr>
<td>2</td>
<td>95%</td>
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<td>95%</td>
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<td>55%</td>
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<tr>
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<td>71%</td>
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### Table IV
Assembly protocol used in preparing Sample Set #4

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<tr>
<th># of Samples</th>
<th>% PEG4 (v/v)</th>
<th>% C18 (v/v)</th>
<th>% C12 (v/v)</th>
<th>Assembly Volume</th>
<th>Assembly Temperature</th>
<th>Assembly Time</th>
<th>Post Soak</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>1 hour</td>
</tr>
<tr>
<td>2</td>
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<td>0%</td>
<td>0%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>1 day</td>
</tr>
<tr>
<td>2</td>
<td>55%</td>
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<td>Room Temp</td>
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<td>No</td>
</tr>
<tr>
<td>2</td>
<td>55%</td>
<td>0%</td>
<td>45%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>1 hour</td>
</tr>
</tbody>
</table>

*Assembled on a 4 cm x 2.5 cm x 0.1 cm ultra smooth glass microscope slide coated with a 10 Å Cr adhesion layer and 500 Å gold via electron beam evaporation. Assembly done in a sealed polystyrene petri dish.*

### Table V
Assembly protocol used in preparing Sample Set #5

<table>
<thead>
<tr>
<th># of Samples</th>
<th>% PEG4 (v/v)</th>
<th>% C18 (v/v)</th>
<th>% C12 (v/v)</th>
<th>Assembly Volume</th>
<th>Assembly Temperature</th>
<th>Assembly Time</th>
<th>Post Soak</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
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<td>0%</td>
<td>0%</td>
<td>4 mL</td>
<td>60 °C</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>55%</td>
<td>45%</td>
<td>0%</td>
<td>4 mL</td>
<td>60 °C</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>55%</td>
<td>0%</td>
<td>45%</td>
<td>4 mL</td>
<td>60 °C</td>
<td>1 hour</td>
<td>No</td>
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### Table VI
Assembly protocol used in preparing Sample Set #6

<table>
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<tr>
<th># of Samples</th>
<th>% PEG4 (v/v)</th>
<th>% C18 (v/v)</th>
<th>% C12 (v/v)</th>
<th>Assembly Volume</th>
<th>Assembly Temperature</th>
<th>Assembly Time</th>
<th>Post Soak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>4 mL</td>
<td>65 °C</td>
<td>1 hour</td>
<td>No</td>
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<tr>
<td>1</td>
<td>0%</td>
<td>100%</td>
<td>0%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>0%</td>
<td>0%</td>
<td>100%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>85%</td>
<td>15%</td>
<td>0%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>85%</td>
<td>15%</td>
<td>0%</td>
<td>4 mL</td>
<td>65 °C</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>55%</td>
<td>0%</td>
<td>45%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>55%</td>
<td>0%</td>
<td>45%</td>
<td>4 mL</td>
<td>65 °C</td>
<td>1 hour</td>
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<tr>
<td>1</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>4 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>1 hour</td>
</tr>
<tr>
<td>1’</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>8 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1’</td>
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<td>100%</td>
<td>0%</td>
<td>8 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
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</tr>
<tr>
<td>1’</td>
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<td>0%</td>
<td>100%</td>
<td>8 mL</td>
<td>Room Temp</td>
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<td>No</td>
</tr>
<tr>
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<td>0%</td>
<td>8 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1’</td>
<td>55%</td>
<td>0%</td>
<td>45%</td>
<td>8 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>No</td>
</tr>
<tr>
<td>1’</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>8 mL</td>
<td>Room Temp</td>
<td>1 hour</td>
<td>1 hour</td>
</tr>
</tbody>
</table>

*Assembled on a 4 cm x 2.5 cm x 0.1 cm ultra smooth glass microscope slide coated with a 10 Å Cr adhesion layer and 500 Å gold via electron beam evaporation. Assembly done in a sealed polystyrene petri dish.*
preparing sample sets 1-6, refer to Tables 1-6.

2.2 SAM Surface Analysis

2.2.1 Electron Spectroscopy for Chemical Analysis (ESCA)

A Surface Science Instruments S-Probe spectrometer was used to acquire ESCA data. This instrument is equipped with a monochromatized aluminum Ka x-ray source and a hemispherical analyzer. Survey and detail scans were acquired at a pass energy of 150 eV to determine sample composition. High resolution spectra were acquired at a 50 eV pass energy. Analysis was carried out using the ESCAVB data reduction software. Due to time constraints, ESCA analysis was limited to two spots per sample. As a result, all data reported are averages but because n=2, standard deviations could not be calculated. Typical errors for the ESCA (C+O)/Au ratio and composition calculations for SAMs is ± 5% [3].

2.2.2 Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS)

ToF-SIMS spectra were acquired on a Physical Electronics PHI 7200 Time-of-Flight Spectrometer using an 8-keV Cs+ ion source in the pulsed mode. Spectra were acquired for both positive and negative secondary ions over a mass range of m/z = 0 to 1000. The ion source was operated with a pulse width of 1.2 ns and a current of 1.5 pA. Secondary ions of a given polarity were extracted into a two stage reflectron Time-of-Flight Mass Analyzer and detected using a pair of stacked micro channel plates equipped with a time to digital converter. Spectra were acquired using an analysis area of 0.01 mm² and the total ion dose was maintained below 2 x 10¹² ions/cm². Negative spectra were calibrated using the CH⁻, C₂H⁻, and AuS⁻ peaks. Positive spectra were calibrated using the CH₃⁺, C₂H₅⁺, and AuSCH₂⁺ peaks. Calibration errors were below 10 ppm. Mass resolution (m/Δm) for a typical spectrum was between 5000 and 6000. All ToF-SIMS data reported are averages without standard deviations due to the limiting n=2. Typical error on ToF-SIMS composition calculations for SAMs is ± 2% [3].

3. RESULTS AND DISCUSSION

3.1 Surface Percent PEG4

ESCA analysis showed only the presence of the expected elements (C, Au, S, and O) on SAM surfaces. High resolution S 2p scans showed only the presence of bound sulfur, with no oxidation. Because no oxidized sulfur was present, it could be assumed that all oxygen on the surface was from the PEG4 thiols. Using this assumption, the percent PEG4 on the SAM surface was estimated using the percent oxygen found from ESCA analysis and Equation 1. The factor of 3.8 in Equation 1 is the ratio of carbon atoms to oxygen atoms in the PEG4 thiol chain.

\[
\%\text{PEG}_{\text{surface}} = 3.8 \left( \frac{\%O_{\text{surface}}}{\%C_{\text{surface}}} \right)
\]

The percent of PEG4 thiols on a SAM surface can also be calculated from ToF-SIMS data. This calculation can be done using the intensities of the characteristic negative molecular ion peaks from each thiol in the monolayer (shown in Table 7) and Equation 2.

\[
\%\text{PEG}_{\text{surface}} = \frac{\sum \text{PEG}_{\text{ions}}}{ \sum \text{PEG}_{\text{ions}} + \sum \text{CH}_3_{\text{ions}}}
\]

<table>
<thead>
<tr>
<th>TABLE VII</th>
<th>Characteristic negative molecular ion peaks found in ToF-SIMS analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS(CH₂₉CH₃</td>
<td>HSO(CH₂₁₉OCH₂₉CH₂∞OH</td>
</tr>
</tbody>
</table>
yield of PEG4 molecular ions caused by chain entanglement within the PEG4 regions of the surface. The decrease in molecular ion detection of the PEG4 is not equally observed for the CH₃ thiols. This may suggest SAM surfaces are phase segregated and the chain entanglement is occurring primarily in PEG4 regions. Further work would need to be done to verify if phase segregation and chain entanglement are truly involved in this effect. Mixed molecular ion fragments \{\text{Au}[S(CH₂)ₙCH₃][S(CH₂)ₐ](OCH₂CH₂)₄OH]\} in ToF-SIMS could possibly be used for this determination.

3.2 (C+O)/Au Ratio

The ESCA C/Au ratio has been used for SAM surfaces to estimate the thickness of the monolayer films [4,7]. When the monolayer contains PEG4 thiols, the ratio can be revised slightly to account for the oxygen atoms in the thiol chain and becomes (C+O)/Au. Previous work in our laboratory has generated the data shown in Table 8, which was used as a reference for the ESCA analysis performed on these SAM surfaces [3]. The value for an ideal, ordered layer of HS(CH₂)₉(OCH₂CH₂)₄OH (~24 atoms) was estimated based on this data (Table 8). This value can be used to compare the experimental (C+O)/Au ratios of mixed PEG4 SAMs with an ideal ordered PEG4 surface.

Using data obtained from ESCA analysis, the (C+O)/Au ratio was calculated for all mixed PEG4/C18 SAMs and can be seen in Figure 2. The (C+O)/Au ratio for the mixed surfaces suggests that these layers are significantly thicker than a pure, disordered PEG4 layer, but not as thick as expected for a purely ordered layer. It is interesting to note that the mixed surfaces have (C+O)/Au ratios similar to the C18 used in the mixtures. Similar trends were seen for mixtures with the C12 thiol, where the experimental (C+O)/Au ratios were similar to those for pure C12 (data not show). This may suggest that surrounding methyl thiols act as supports for the disordered PEG regions.

<table>
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<th>Chain Length</th>
<th>C/Au</th>
<th>St dev</th>
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<td>0.01</td>
</tr>
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<tr>
<td>24*</td>
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*Estimated value based on the experimental C6 to C18 data [3].

<table>
<thead>
<tr>
<th>vol % PEG4 in solution</th>
<th>vol % methyl thiol in solution</th>
<th>surface % PEG4 from ESCA</th>
<th>avg. (C+O)/Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>82%</td>
<td>13% C18</td>
<td>54.7%</td>
<td>3.62</td>
</tr>
<tr>
<td>55%</td>
<td>45% C12</td>
<td>58.4%</td>
<td>2.01</td>
</tr>
</tbody>
</table>
Because the data in Figure 2 indicates that the mixed PEG4/C18 monolayers remain disordered, it was proposed that using a thiol with a length similar to the alkane portion of the PEG4 thiol might be more effective. This type of technique has been previously applied to the assembly of long DNA chains and chemically altered PEG chains [9,10]. The C18 thiol was originally chosen because of its similarity in length with the entire PEG4 thiol chain. However, since the (C+O)/Au ratios of the mixed surfaces never exceeded that of pure C18, it was hypothesized that the C18 may have been contributing to, rather than decreasing, the disorder of the system. Thus, mixed surfaces of PEG4 and C12 thiol were assembled because the chain length of the C12 thiol matches the chain length of the alkane portion of the PEG4 chain.

Data in Table IX shows a comparison of mixed PEG4 SAMs created with the C18 and C12 thiols. With approximately the same percentage of PEG4 on the SAM surface, the order is significantly less in the C12 mixed SAMs. This is highlighted by the lower (C+O)/Au ratio in the mixed C12 SAMs, which is almost equivalent to that of a pure C12 SAM (1.93). This data led to the conclusion that the problem may not be with the length of the methyl thiol but the way in which SAMs are assembled.

It has been previously hypothesized by Chen et al. that assembly protocol affects the phase segregation of SAM surfaces [2].

3.4 Assembly Time Dependence

Sample Set 2 was designed to illustrate the length of assembly time's affect on the composition of the resulting monolayer. The data for this sample set can be seen in Figure 3. Assembly time data suggests that after 1 h., a phenomenon is taking place that reduces the number of PEG4 thiols that are attached to the Au surface. This is consistent with data found previously in our labs that
showed a similar trend with pure PEG4 surfaces (Graham et. al. unpublished data.)

3.5 Assembly Temperature Dependence

Sample Set 5 was made to understand the assembly temperature’s affect on the resulting order of PEG4 SAMs. It has been reported that assembling mixed SAMs at higher temperatures reduces phase segregation [2]. The data for this sample set can be seen in Figure 4. Temperature data shows that with the same amount of PEG4 in the monolayer, samples assembled at higher temperature are more ordered (i.e. higher (C+O)/Au ratios). This trend may suggest that there is less phase segregation in the high temperature samples. Therefore, the CH$_3$ terminated thiols are better able to increase the order of the surrounding PEG4 thiols. It is also noted that the pure PEG4 samples assembled at high temperature show a higher (C+O)/Au ratio.

3.6 Other Assembly Protocols

In order to further investigate PEG4 SAM surfaces, alternate assembly protocols were experimented with. Instead of mixing the thiol solutions, samples were allowed to assemble in pure PEG4 solution, removed, and then put into a pure CH$_3$ terminated thiol solution for a post soak. A similar procedure has been used previously to stabilize thiolated DNA probes on gold surfaces [9]. Selected data from the PEG4 monolayers are shown in Table X.

Using a post soak of pure CH$_3$ thiol following assembly in pure PEG4 solution seems to increase the order of the system relative to assembling in a mixed thiol solution. This may be due to a decrease in phase segregation of the thiols on the gold surface since the methyl thiols would either be filling in spaces between the PEG4 thiols or displacing the PEG4 off of the surface.

To determine which mechanism is predominant, a post soak was carried out on a SAM created from a mixed solution (Table XI). Because the percent of PEG4 on the SAM surface did not change significantly with the post soak, it is likely that the CH$_3$ thiols were filling in the spaces between PEG4 thiols chains.

The data shown in Table XII was collected to demonstrate the effect of the length of the post soak on the order and composition of the monolayer. Increasing the length of the post soak in CH$_3$ thiol solution decreases both the percentage of PEG4 thiols in the monolayer and the order of the system. It is interesting to note that the sample that was post soaked for one hour in C12 had a similar surface percentage of PEG4 as the sample post soaked in C18 for the same length of time (Table 10). This further supports the conclusion that with a one hour post soak, the methyl thiols are filling in spaces left by the PEG4 thiols. The decrease in PEG4 on the surface after a one day post soak suggests that between one hour and one-day all the available spaces on the gold surface have been filled by the C12 molecules. Further assembly time results in replacement of PEG4 chains by C12 thiols.

### Table X

<table>
<thead>
<tr>
<th>vol. % PEG4 in solution</th>
<th>Post soak</th>
<th>Surface % PEG4 from ESCA</th>
<th>avg. (C+O)/Au</th>
</tr>
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<tbody>
<tr>
<td>50%</td>
<td>no</td>
<td>14.7%</td>
<td>2.78</td>
</tr>
<tr>
<td>100%</td>
<td>1 hour C18</td>
<td>74.2%</td>
<td>3.49</td>
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<tr>
<td>95%</td>
<td>no</td>
<td>77.5%</td>
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### Table XI

<table>
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<tr>
<th>vol. % PEG4 in solution</th>
<th>vol. % C12 in solution</th>
<th>Assembly time</th>
<th>Post soak</th>
<th>Surface % PEG4 from ESCA</th>
<th>avg. (C+O)/Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>55%</td>
<td>45%</td>
<td>1 hour</td>
<td>no</td>
<td>58.4%</td>
<td>2.01</td>
</tr>
<tr>
<td>55%</td>
<td>45%</td>
<td>1 hour</td>
<td>1 hour C12</td>
<td>50.3%</td>
<td>2.10</td>
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</table>

### Table XII

<table>
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<tr>
<th>vol. % PEG4 in solution</th>
<th>Assembly time</th>
<th>Post soak</th>
<th>Surface % PEG4 from ESCA</th>
<th>avg. (C+O)/Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>1 hour</td>
<td>1 hour C12</td>
<td>73.7%</td>
<td>2.35</td>
</tr>
<tr>
<td>100%</td>
<td>1 hour</td>
<td>1 day C12</td>
<td>56.5%</td>
<td>2.08</td>
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</table>
4. CONCLUSIONS AND FUTURE WORK

Based on the data collected, the combination of the following factors promotes order in mixed PEG4 monolayers: longer CH₃ terminated thiols [(SH(CH₂)₁₁CH₃) rather than (SH(CH₂)₁₇CH₃)], approximately a 50/50 PEG4/CH₃ thiol ratio in the monolayer, higher assembly temperatures (60 - 65°C), and shorter assembly time (one hour rather than 2 days). Further work is in progress to better understand the factors that contribute to the order in a mixed poly(ethylene glycol) self-assembled monolayer.

Selected surfaces are currently being studied using near edge X-ray Adsorption Fine Structure Spectroscopy (NEXAFS) and Surface Plasmon Resonance (SPR). These supplementary analysis techniques will provide additional structural and fouling behavior information, respectively, about the SAMs. Future work may also include chemical modification of the PEG4 thiol to induce ordering by increasing chain interactions in the monolayer.

ACKNOWLEDGEMENTS

Special thanks to Dan Graham for his support and guidance. Funding provided by UWEB (NSF EEC 95291610), and NESAC/BIO (NIH Grant RR-01296). Additional thanks to Esmaeel Naeemi for thiol synthesis, Jeff Schwartz for substrate preparation, and Stephen Golledge and Matt Wagner for instrument and software assistance.

REFERENCES

The Effects of Laminin 5 Coating on Keratinocyte Migration along Percutaneous Devices

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Abstract: Extensive epithelial migration along percutaneous devices is a significant factor in device failure. Laminin 5 is an adhesion protein important in regulating epithelial cell migration during epidermal wound healing. In this study, polypropylene sutures, approximately 200µm in diameter were used as a model percutaneous device. The surfaces of the sutures were coated with either laminin or fibronectin. Laminin-coated, fibronectin-coated and uncoated sutures were implanted through the dorsal skin of C57 black mice. After three days, the implant area was biopsied and prepared for microscopy. The distance of epithelial migration along each implant was then measured. Preliminary data suggests that the laminin-coated devices show a shorter distance of epithelial migration than do the fibronectin-coated and uncoated sutures.

We hypothesize that the presence of laminin 5 on the surface of a percutaneous device will prevent epithelial downgrowth along the device by anchoring migrating keratinocytes to the implant. In this experiment, protein-coated devices were implanted through the dorsal skin of C57 black mice. The implant and surrounding tissue was later explanted and prepared for microscopy. The distance of keratinocyte migration along each device was then measured.

1. INTRODUCTION

Percutaneous devices are objects that permanently penetrate through a defect in the skin. Because they penetrate into and through a surgically-created opening, percutaneous devices provide a connection between the interior and exterior of the body. They are designed for a variety of clinical and biomedical research applications including catheters, middle ear ventilating tubes, and dental implants [1].

A permanent seal of the integument around the device is essential for functionality. Therefore, a limiting factor in the success of a percutaneous device is the body’s complex healing mechanism. After implantation, epithelial cells called keratinocytes aggressively migrate downward along the device, eventually forming a sack around the implant and extruding it [1]. Because of this behavior, most experimental devices are likely to fail within a few weeks in the absence of heroic measures [2].

Wound healing involves both epidermal proliferation and the rebuilding of connections between the skin’s dermal and epidermal layers. These two layers are held together by a complex network of interconnecting proteins called the dermal-epidermal junction (DEJ). DEJ proteins include laminins, collagens and integrins. Laminin 5 has been shown to directly regulate adhesion, spreading and motility in epithelial cell types in the following way: upon injury to the epidermis, a precursor form of laminin 5 is transcribed and deposited into the dermal wound bed. This precursor laminin 5 is then cleaved by an endogenous protease, releasing mature laminin 5 as well as two peptides. The presence of mature laminin 5 in the wound bed signals the keratinocytes at the wound edge to migrate over the exposed dermal collagen and close the wound. Laminin 5 then anchors keratinocytes to the lower collagen layer [3, 4].

2. MATERIALS AND METHODS

2.1 Implants

Polypropylene sutures (2-0 prolene, 8685 from Ethicon) approximately 200µm in diameter were used as model percutaneous devices. The sutures were coated with human laminin 5, murine laminin or fibronectin before being implanted. Uncoated sutures were also implanted. Sutures were purchased with a cutting needle attached.

2.2 Preparation of Control Sutures

Because laminin 5 is not available for purchase, Human Foreskin Keratinocytes (HFK) were obtained and cultured. Human Foreskin Keratinocytes were then seeded onto the polypropylene sutures, where they spread and deposited
laminin 5. HFK’s can then be removed with ammonium hydroxide, leaving only protein behind on the suture’s surface.

2.3.1 Cell Culture

HFKs were obtained from the laboratory of Professor John Olerud at the University of Washington. Cells were cultured in serum-free EpiLife medium (Cascade Biologicals, Inc. Portland, OR). The medium was supplemented with calcium and Human Keratinocyte Growth Supplement (Cascade Biologicals, Inc. Portland, OR). Cultures were maintained at 37°C under 5% CO₂ conditions. Media was replaced every 2-3 days. Cells were passaged at 60-80% confluence using Trypsin/EDTA and Trypsin Neutralizer solutions (Cascade Biologicals, Inc. Portland, OR).

2.3.2 Seeding Sutures with HFK’s

Several methods were devised to induce HFK’s to adhere to and spread on polypropylene sutures.

Method 1: Three sterile sutures were suspended across a 25 mm diameter stainless steel Leiden Dish. The Leiden Dish was placed in a 50 mm tissue-grade polystyrene dish. HFK’s (3 x 10⁶) were suspended in 5 mL of medium and deposited into the center of the Leiden Dish. The dish was then covered and incubated at 37°C under 5% CO₂ conditions. After 2 days, sutures showed minimal cell adhesion. Cells were found to preferentially absorb to the polystyrene dish. After 4 days, rust was discovered on the Leiden dish and the culture was discarded.

Method 2: Three sterile sutures were coiled in separate 35 mm non-tissue grade polystyrene dishes. A non-tissue grade polystyrene surface has fewer cell adhesion sites than a tissue-grade surface and was used in an attempt to reduce preferential cell attachment to polystyrene. Cells (1 x 10⁶) suspended in 3 mL of medium were deposited into each dish. Dishes were covered and incubated at 37°C under 5% CO₂ conditions. Fewer cells were adhered to the dish. However, there was not significantly higher number of cells observed on the surface of the sutures.

Method 3: Sutures were placed in 15mL centrifuge tubes containing 15mL of media and 1.5 x 10⁶ cells. In order to further prevent preferential cell adhesion to the polystyrene, the tubes were tumbled at 3 revolutions per minute by a 16 inch diameter tissue culture rotator. The machine operated in a 37°C, 5% CO₂ incubator for 2 days. The moving cells did not adhere to the tube. However, minimal adhesion to the polypropylene suture was observed.

Method 4: To increase the number of binding sites on the surface of polypropylene, sutures were treated with acetone in a plasma reactor prior to being seeded with HFKs. On the surface of the sutures, the plasma treatment produced hydroxyl and carboxyl groups to which HFKs could attach.

Three segments of plasma-treated suture were taped to the bottom of a 50 mm non-tissue culture grade polystyrene dish. Cells (2 x 10⁶) suspended in 5 mL medium were deposited into the dish. A significantly higher number of cells adhered to the sutures than had been previously observed. Therefore the acetone plasma treatment was successful in increasing cell adhesion to polypropylene.

Due to fungal contamination the HFK- coated sutures were discarded. Therefore, we were unable to produce laminin 5 coated sutures. The experiment was performed using laminin-coated, fibronectin-coated and uncoated sutures.

**Figure 1**
Epithelial migration along an uncoated implant. Clot forms at entrance wound (arrow 1) and migrating keratinocytes (arrow 2) travel a distance of 592µm down the device.

**Figure 2**
Epithelial migration along an uncoated implant. Clot forms at entrance wound (arrow 1). The thickness of the migrating epithelial layer (arrow 2) is 39µm.
All animals were used according to protocols approved by the University of Washington’s Institutional Animal Use and Care Committee.

Sutures were implanted through the dorsal skin of C57-black mice weighing 20-26 g. The mice were anesthetized with a ketamine/xylazine mixture diluted in saline. The fur on the dorsal surface was shaved and treated with depilatory, and the underlying skin cleaned with Betadine. The needle and suture were pulled horizontally through a section of skin to create percutaneous sites at both the entrance and exit wounds. The sutures were then secured flat against the backs of the mice with Tegaderm™wound dressing (3M Corp. St. Paul, MN).

Three sutures were implanted into each of three mice, creating 6 percutaneous sites per animal. In each animal, one suture was laminin-coated, one fibronectin-coated and one uncoated. The implants remained in vivo for three days.

The mice were sacrificed by narcotic overdose. Eight sites were found intact: three laminin sites, two fibronectin sites and three uncoated sites. Four implants were completely absent. Two implants were partially removed, only one end of the suture remained implanted in the skin.
findings suggest that additional means of securing the implants is necessary to prevent undue implant movement or loss.

2.4 Histology and Microscopy

The implants and surrounding tissue were removed and placed into Karnovsky’s fixative overnight. Samples were then dehydrated and embedded in wax. Sections were cut obliquely along the length of the suture, stained with Hematoxylin and Eosin and examined under a Nikon E800 microscope. Images were recorded using 4x/.2 and 10x/.45 plan apo objectives and a Photometrics CoolSNAP color digital imager.

3. RESULTS

Epithelial migration was found on uncoated, laminin-coated, and fibronectin-coated implants. The epithelial layer traveled nearly the entire length of the uncoated implants (Fig. 1). Figures 1 and 2 show the migrating layer to be 592µm in length and 39µm thick.

Keratinocyte migration along laminin-coated implants was found to be shorter than migration along uncoated implants. The migrating epithelial layer covered only small portions of the total lengths of the devices (Fig. 3 and 5). Figure 3 shows a 60µm thick (Fig. 4) keratinocyte layer to have traveled 343µm down the pictured implant. On a second laminin-coated device, the 49µm thick (Fig. 6) layer spans 339µm (Fig. 5).

All fibronectin-coated devices were lost. However, the tissue surrounding the implant site was explanted and prepared for microscopy in a manner similar to the other samples. Keratinocytes were found to have migrated down the entire length of the entrance wound. The migrating layer was 774µm in length (Fig. 7) and 63µm thick (Fig. 8).

4. DISCUSSION

The length of epithelial migration is inversely proportional to the success of the device. Therefore, devices showing a short migrating epithelial layer are more likely to remain intact and functioning. While keratinocytes migrated down the entire length of the uncoated device, they traveled only a short distance down the laminin-coated device. Therefore, laminin surface coatings could lengthen the lifetime of a percutaneous device.

Although we were able to measure the distance of epithelial migration along laminin-coated and uncoated devices, these measured distances cannot be directly compared. During sectioning, tissue samples were cut at different angles through the suture. Therefore, the laminin-coated and un-
coated images show different orientations around the suture. This inconsistency could be remedied in future experiments by placing the sutures perpendicular at the time of implant and anchoring them at a uniform orientation during the implant period and sectioning. Despite variation in orientation, a trend of shorter epithelial migration along laminin-coated devices and longer migration along uncoated devices can be seen.

In the current study, we were unable to coat our devices with human laminin 5. Factors limiting the success of our current methods include: (1) lack of available adhesion sites on the surface of polypropylene sutures; and (2) lack of a sterilizable suspension device. It was observed that acetone plasma treatment increases the number of cells that will adhere to polypropylene sutures. If combined with the use of an effective suspension device (Fig. 9), acetone plasma treatment could be successful in attracting a sufficient number of adhered HFKs to the surface of a polypropylene suture to create a laminin 5 surface coating.

The relative success of the laminin-coated implants compared with uncoated implants suggests that coating the surface of a percutaneous device with laminin 5 will significantly reduce epithelial migration. If implants could be efficiently coated, the lifetime of devices such as catheters, peritoneal dialysis access ports and glucose sensors could potentially be increased. The current work did not address other factors in epithelial migration such as surface topography, other adhesion proteins and growth factors. It may be useful to combine these factors and laminin surface coating in future experiments.

5. CONCLUSION

Acetone plasma treatment significantly increased the number of HFK that adhered to and spread on the surfaces of polypropylene sutures. Laminin-coated, fibronectin-coated and uncoated sutures were implanted into a mouse model. Laminin-coated implants showed shorter epithelial migration than did the uncoated implants.

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REFERENCES

Cell Patterning On Silicon-Gold Chips Using A Highly Selective Protein Patterning Technique

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Abstract: Crucial to the development of biomedical microdevices is a protein patterning technique that retains protein bioactivity and guides natural cell growth on solid surfaces. We have used a highly selective protein patterning method based on photolithography and chemical selectivity to guide cell growth on solid substrates. Micro-arrays of gold in the shape of squares or strips were fabricated on silicon substrates using photolithography. The gold regions were modified with mixed – COOH-terminated self-assembled monolayers (SAMs) to have a high affinity for desired proteins, while silicon regions were coated with polyethylene glycol (PEG) to repel proteins. Fibronectin was immobilized on SAM layers by use of a N-Hydroxysuccinimide (NHS) conjugate. Fluorescent-labeled fibronectins were synthesized and immobilized on the patterned surfaces to characterize the proteins’ locations and level of coverage on arrays by fluorescent microscopy. To investigate the ability of PEG for passivation of silicon regions, same surfaces were treated by fluorescent–labeled fibronectin but with no PEG modification. Results revealed that proteins were selectively immobilized and uniformly covered the gold patterns of PEG treated samples, while randomly spread on non-PEG treated surfaces. To investigate the ability of the proposed technique for cell adhesion, NIH 3t3 fibroblast cells and human HeLa cells were cultured on strip-patterned surfaces for 24 hours. Results indicated that cells adhered preferentially to gold regions and grew along the strips’ direction. This technique is simple, highly selective and adaptable to large-scale applications. The protein patterning approach has also a great potential for nano-scale patterning.

1. INTRODUCTION

Cellular adhesion and growth on solid surfaces play an important role for fundamental studies of cell biology and for novel technological applications that employ cellular patterns, including cell-based biosensors, diagnostic devices and more recently tissue engineering products [1-3]. Successful cellular patterning relies on the existence of: (1) a versatile, and reproducible micro-fabrication method; (2) a highly selective protein patterning technique for natural guided cell growth that has no deleterious effect on the bioactivity of biomolecules (proteins and cells); (3) a stable and robust surface modification for both cell adhesion promoting and cell rejecting regions of the surface; (4) a cell delivery process that has no damage on cell functionality or morphology during or after cell adhesion; and (5) a versatile method for application of different types of cells.

Design of a technique that can address all of these requirements is a challenge. Other techniques that have been developed so far, including metallic stencils [4], microfluidic channels [5,6] and elastomeric stencils [7], are lacking at least one of the mentioned parameters.

We have combined photolithography and chemical selectivity to pattern non-denatured proteins on gold-silicon substrates to guide cell adhesion. Selective cell attachment is achieved by microfabricating a patterned substrate to which proteins and cells adhere preferentially. The microfabrication process can create accurate gold patterns with different shapes and sizes on silicon substrates, inexpensively. The substrate surface is modified such that the gold regions have a high affinity for desired proteins, while silicon regions were coated with polyethylene glycol (PEG) to repel proteins. Fibronectin was immobilized on SAM layers by use of a N-Hydroxysuccinimide (NHS) conjugate. Fluorescent-labeled fibronectins were synthesized and immobilized on the patterned surfaces to characterize the proteins’ locations and level of coverage on arrays by fluorescent microscopy. To investigate the ability of PEG for passivation of silicon regions, same surfaces were treated by fluorescent–labeled fibronectin but with no PEG modification. Results revealed that proteins were selectively immobilized and uniformly covered the gold patterns of PEG treated samples, while randomly spread on non-PEG treated surfaces. To investigate the ability of the proposed technique for cell adhesion, NIH 3t3 fibroblast cells and human HeLa cells were cultured on strip-patterned surfaces for 24 hours. Results indicated that cells adhered preferentially to gold regions and grew along the strips’ direction. This technique is simple, highly selective and adaptable to large-scale applications. The protein patterning approach has also a great potential for nano-scale patterning.
2. MATERIALS

The following materials were used as received: 11-mercaptoundecanoic acid 95% (11-MUA), 3-mercaptopropionic acid 99% (3-MPA), N-hydroxysuccinimide (NHS), 1-ethyl-3-dimethylamino-propyl carbodimide (EDAC), polyethyleneglycol-silane (PEG, MW 5000) from Shearwater Polymers (Huntsville, AL), fibronectin (Tetramethyl Rhodamine), sigmacote, and phosphate buffer powders from Sigma (St. Louis, MO). Carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE) from Molecular Probes (Eugene, OR) RPMI media and trypsin were obtained from American Tissue Culture Collection (ATCC, Manassas, VA). All ethanolic solutions were deoxygenated by N\textsubscript{2} before use. All other solvents were purchased from Sigma or ATCC.

3. METHODS

3.1 Surface Fabrication

The processes for fabricating gold patterned silicon substrates are detailed in [8,9]. Briefly, a mask of arrays in the desired shape and size was printed onto a photoresist-covered silicon wafer. A thin layer of titanium (Ti) (5nm) was then deposited onto the photoresist-developed substrate. Gold films were subsequently deposited onto Ti at a deposition rate of 0.3 angstrom/s. The photoresist was then dissolved in acetone and the remaining metal films were lifted off. Finally, the patterned silicon wafers were cut. In order to avoid surface contaminants and unexpected scratches, the silicon wafers were coated with a layer of photoresist of 2 mm in thickness on their polished sides before cutting.

3.2 Surface Modification

In order to remove the photoresist layer and clean the substrates, all the samples were sonicated with acetone (10 min.) and ethanol (5 min) followed by DI wash. The substrates were then placed into a piranha solution containing hydrogen peroxide and sulfuric acid (H\textsubscript{2}O\textsubscript{2}/H\textsubscript{2}SO\textsubscript{4} 2/5 (v/v)) for further cleaning and formation of oxide layers on silicon regions. To remove any acid residue from the surface, the samples were extensively washed with DI water. Clean samples were then transferred to a container under the nitrogen stream in order to be passively dried.

The COOH- terminated alkanethiol formation was achieved by placing each of the samples in a 20 mM ethanolic solutions mixed 11-MUA and 3-MPA (1 to 10 v/v) for 16 hours. To have the NHS attach to the terminating COOH, the samples were placed in an aqueous solution of EDAC (150 mM) and NHS (30 mM) for 30 minutes.

The gold-silicon chips were then placed in a PEG-silane solution. All the glassware for PEG synthesis was coated by sigmacote solution to eliminate any PEG-silane reaction with the glass surface. The solution of 10 mg/ml was prepared by adding PEG-silane in Toluene and 50 mg/ml of triethyl amine as the catalyst. Each chip was then placed in a glass bulb covered in PEG solution and refluxed with nitrogen gas for 4 hours. After being taken out, the substrates were immersed in a toluene solution and sonicated for 10 minutes. Then each was rinsed with ethanol and DI water and dried passively with nitrogen.

At this point the gold portion of the surfaces had SAM attached to the NHS group and the silicon attached to the PEG layer (Fig. 1). Protein was attached to the gold substrate through a 45-minute exposure of the samples to the protein solution (0.1 mg/ml, PBS with pH 8.2).

It should be mentioned that pure fibronectin protein was used for cell culture, but (5) and (6)-TAMRA- Fibronectin was synthesized and used for fluorescent microscopy.

3.2.1 Fluorescence Labeling Method

Fibronectin proteins were diluted with PBS buffer (pH 8.2) to 0.1 mg/ml solutions. (5(6)-TAMRA, SE) labels were dissolved in DMF to prepare 5 mg/ml solutions. Labeling reaction was performed (Fig. 3) by adding 3 ml of Rhodamine solution to the 6 ml of Fibronectin samples that were shaking at room temperature. The mixture was stirred in dark for one hour at room temperature. The resulting solution was dialyzed extensively against PBS buffer (pH 8.2) using a spectrum dialysis membrane (cutoff molecular weight < 10,000) at 4°C in order to remove unreacted molecules.

![REACTION 1](attachment:image.png)

The reaction between Rhodamine label and amine terminus of fibronectin. Two arrows in carbon 5 and carbon 6 positions show that Rhodamine label is a mixed isomers of 5 (and-6)-carboxytetramethylrhodamine, succinimidyl ester.
3.3 Cell Culture

Selective adhesion of cells to the protein-immobilized surfaces happened through culture of NIH 3T3 fibroblast cells and human HeLa cells for 24 hours. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ (in air), in 75 cm² flasks containing 10 ml of relevant media supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin. RPMI-1640 medium was used for HeLa cells, while DMEM used for NIH-3T3 fibroblast cells. Media was changed every third day. Subconfluent cultures of cells were dissociated using a trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA). Cell dissociation was carried out by two times PBS wash, 15 seconds tripsinization and 5-10 min incubation at 37°C. A fresh media were then added in the flask at room temperature to inhibit the effect of trypsin. Cell viability was determined through staining with Trypan Blue and cell density was estimated using a 0.9 mm³ counting chamber and a hemocytometer. Generally, 0.5 ml of each cell type were plated on to the modified substrates at a density of 10⁵ cells/ml and were allowed to adhere for 24 hours under standard culture condition. Adherent cells were then fixed with 2% glutaraldehyde for 20 minutes at room temperature. Cell patterned substrates were then visualized by optical reflectance microscopy.

4. RESULTS AND DISCUSSION

We have combined photolithography and chemical selectivity to pattern non-denatured proteins capable of guiding cell adhesion on solid substrates. Precise patterns of gold in different shapes are cheaply made on silicon substrates by photolithography. The high selective protein patterning is accomplished by modifying the gold regions with the functional groups with a strong affinity for proteins/cells and the silicon regions with protein resistant films such as PEG. PEG modification provides biocompatible layers on silicon regions. PEG has been used most widely for surface modification because of its unique properties such as hydrophilicity, flexibility, high exclusion volume in water, nontoxicity, and nonimmunogenicity [10]. As a result the modification scheme shown in Figure 1 provide biocompatible coatings on both regions, which have no adverse effect on protein and cell functionalities. In this approach, protein immobilization is the last step of the protein patterning process so that the natural conformation of proteins can be retained when they are attached to the gold regions of the surface. Also, the covalent immobilization of fibronectin via N-hydroxysuccinimide conjugate forms a robust layer on the surface. Successful attachment of the protein relies on the accessibility of its primary amino groups to the terminal

**Figure 1**

Chemical reaction sequence for coating the patterned surface with proteins on Au and PEGs on Si regions followed by cell attachment [9].

**Figure 2**

Fluorescent images of the patterned substrates: (A) after PEG surface modification for the silicon region and fibronectin immobilization on gold regions of square-patterned substrates, (B) Only fibronectin immobilization on gold regions of square-patterned substrates, (C) same condition as (A) on strip patterned substrates, and (D) same condition as (B) on strip-patterned substrates.
groups of the NHS layer. This means that fibronectin has to come into direct contact with NHS layer. Fibronectin is a 440 KD (Kilo Dalton) glycoprotein containing RGD-sequence (Arg-Gly-Asp tripeptode) [11]. Both Arginine and Asparagine have functional groups that can be readily involved in covalent bond formation.

Figure 2 shows fluorescent images of the gold-patterned silicon substrate PEG surface modification (Fig. 2A and 2C) and without PEG surface modification (Fig. 2B and 2D). Both Figure 2A and Figure 2C show that the silicon regions of the substrates coated with PEG exhibited nearly complete protein reduction. Figure 2B and Figure 2D not only suffer from physically adsorbed fibronectin on silicon regions, but also from an insufficient protein immobilization on gold regions as compared to their PEG modified counter parts. This can be easily attributed to the improved affinity for protein immobilization on the gold regions of the substrates, which contains protein resistant layers (PEG) on the spacing between the patterns. PEG layers have clearly produced passive layers on silicon regions next to highly reactive layers for protein binding on gold regions. On the non-PEG modified surfaces there is no competition between the different regions of substrates.

The optical micrographs in Figure 3 illustrate the successful adhesion of (A) NIH 3t3 fibroblast and (B) human HeLa cells after 24-hour culture with the cell density of 10^5 cells/ml. Both square-patterned and strip-patterned surfaces guided a high selective cell adhesion on gold regions, where fibronectin was immobilized. It is known that binding of fibronectin to cell surface receptors promotes the strength of adhesion, focal contact formation, cytoskeleton formation and cell spreading [12].

This paper presents a convenient method to precisely pattern proteins and cells with high selectivity on micromachined surfaces with the micron-scale resolution. The attached biomolecules retain their bioactivity after the patterning, which is critical for development of high performance microdevices. This patterning technique is versatile and can be used to pattern a variety of proteins, peptides, and other biomolecules with N-terminus on gold-patterned silicon or oxide substrates for guiding variety of cell adhesion. The protein patterning step can also be extended for nano-scale patterning applications.

5. CONCLUSION

Fibronectin arrays were formed on the gold-patterned silicon substrates to guide cell adhesion on those regions. Protein immobilization on alkanethiol SAMs-modified gold regions and PEG binding to the silicon regions of the patterned substrates provided stable biocompatible layers on the substrates. Comparison of PEG modified and unmodified substrates showed that PEG modification improved the coverage of proteins on the gold regions while passivating the silicon regions. Selective adhesion of NIH-3t3 and HeLa cells on fibronectin modified regions of the substrates indicated the ability of patterning technique for guiding cell adhesion on patterned substrates. The proposed method is well adaptable for the development of cell-based microdevices and nano-scale patterning, since protein patterning step is solution based and all regions on the substrates remain intact after cell seeding, thus eliminating the risk of damage to adhered cells and substrates.

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REFERENCES


Stable Surfaces for Diagnostics: Porphyrin Modified Self-Assembled Monolayers as Test Substrates for Fluorescence Detection on Gold

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Abstract: Self-assembled monolayers (SAMs) are being investigated as platforms for DNA microarrays. Hybridization in DNA microarrays is often detected by fluorescence, but gold quenches fluorescent signals making detection problematic or impossible. To combat this, a set of proprietary dyes has been designed to fluoresce on gold surfaces. The goal of this study was to characterize an amine-terminated SAM surface and then use these surfaces as substrates to couple a potentially fluorescent porphyrin molecule. These surfaces were then tested to see if fluorescence could be detected. It was found that amine thiols were difficult to work with, but it was shown that the most highly optimized layer (where the oxygen contamination is greatly reduced and the sulfur oxidation eliminated) is formed with a basic assembly and a basic rinse. Results of the porphyrin reaction and fluorescence detection are pending. Hopefully, these results will help determine the suitability of using SAMs in DNA microarray technology.

1. INTRODUCTION

Diagnostic chips, also called biochips or gene chips, include a set of technologies for characterizing and analyzing large sets of biological data [1-3]. One of the most prominent types of diagnostic chips is the DNA microarray, or DNA chip. The chips consist of DNA sequences that are bound to a surface at precisely defined locations on a grid. The sequences can be either oligonucleotides (approximately 20-25 nucleotide bases), complete or partial cDNA (approximately 500-5,000 nucleotide bases), depending on the analysis required [1-3]. When sample DNA is applied to the array, hybridization will occur at sites with complementary sequences. Successful hybridization is typically detected with radioactive or fluorescent markers. Since the location of the target DNA is known, it is then possible to determine the sequence of the sample DNA. DNA microarray technology provides a means to examine large numbers of molecular changes related to a biological process in a high throughput manner [4].

The diagnostic chip industry makes up a significant part of the multi-billion dollar biotechnology industry. There are several functional biochip strategies that have been developed, but many suffer from reproducibility problems. To address this issue, self-assembled monolayers (SAMs) are being investigated as platforms for DNA microarrays. SAMs are an ideal platform for forming complex engineered surfaces since they easily form reproducible layers. SAMs form when a gold substrate is placed in a solution of thiol (HS(CH₂)ₙX). The sulfur atom at the end of the thiol spontaneously adsorbs to the gold, forming a semi-covalent bond.

The chemistry presented at the outer surface of the SAM can be controlled by the functional group X placed at the end of the thiol chain. Depending on the functionality X, the SAM surface can be further modified by a surface derivatization reaction. Nevertheless, SAM surfaces do face potential obstacles for use in DNA microarrays. For example, hybridization in DNA microarrays is often detected by fluorescence, but gold quenches fluorescent signals making detection problematic or impossible.

For SAM technology to be incorporated into DNA chips, the problem of fluorescence quenching must be addressed. Fluorescent quenching is attributed to the proximity of the dye to the gold surface. The fluorescence quenching mechanism is not very well understood, but is commonly attributed to energy transfer from the electronically excited dye to the gold [5].

To circumvent this drawback of gold, a set of proprietary dyes has been designed to fluoresce on gold surfaces. The goal of this study is to characterize an amine-terminated SAM surface and then use these surfaces as substrates to couple a potentially fluorescent porphyrin molecule (FPM). These surfaces would then be tested to see if fluorescence can be detected.

2. METHODS AND MATERIALS

2.1 Preparation of SAMs

Several sets of SAMs were prepared to optimize the formation of amine-terminated layers.
SAM Set I: Test SAMs were prepared using decane thiol (HS(CH₂)₉CH₃) (Aldrich, 96%) and an amine-terminated thiol (HS(CH₂)₁₁NH₂) (in-house synthesis). The SAMs were assembled using a standard protocol for SAM preparation [6]. Briefly, samples were prepared using pure amine thiol, pure methyl thiol, and a 50:50 (v/v) mixture of the thiols. The substrates used were 1 cm by 1 cm silicon wafers coated with approximately 50 Å of titanium and 1000 Å of gold by electron beam evaporation. 1mM solutions were prepared in 200-proof ethanol (Ricca Chemical Co.). The SAMs were assembled for three days. All samples were washed in ethanol, dried with nitrogen gas, and then sealed in a petri dish. The samples containing the amine thiol were washed with a 10% acetic acid solution in ethanol, then washed again with ethanol before being dried with nitrogen. The samples were then analyzed using ESCA and TOF-SIMS.

SAM Set II: The second set of SAMs was prepared to test various treatments to improve the amine layers. Samples were prepared in acidic, basic, and neutral assembly solutions and rinsed in either acidic or basic solutions. Samples were prepared from 0.2 mM solutions. For the acid assembly, approximately 0.5 mL of trichloroacetic acid (Aldrich, 99%) was added to 40 mL of the thiol solution (pH ~2). The basic assembly was prepared from 40 mL of the thiol and approximately 1.5 mL of ammonium hydroxide (Fischer Scientific, Cert ACS) (pH ~10). The SAMs were assembled for two days, rinsed with ethanol, sonicated in either acidic or basic solution, sonicated in ethanol, rinsed again with ethanol and then dried with nitrogen gas. The acid rinse consisted of a 10% acetic acid solution in ethanol, while the basic rinse was a 5% ammonium hydroxide solution in ethanol. These samples were analyzed using ESCA and TOF-SIMS.

SAM Set III: A third set of SAMs was prepared from mixtures of amine thiol and decane thiol. These samples were made to reduce the number of amines on the surface in case future derivatization was inhibited by steric hindrance. 0.5 mM solutions of each thiol were prepared. Mixtures were made with 90% amine 10% methyl (v/v), and 70% amine 30% methyl (v/v). Approximately 0.9 mL of ammonium hydroxide was added to the final solutions. Assembly time was either 1 h or 3 days. The samples were then rinsed in a 5% ammonium hydroxide solution. These samples were analyzed with ESCA.

SAM Set IV: A fourth set of SAMs was prepared for coupling with the FPM. 0.5 mM solutions of amine, methyl, and hydroxyl thiol (11-mercapto-1-undecanol, HS(CH₂)₁₁OH, Aldrich, 97%) were prepared. Samples were prepared from pure amine, pure hydroxyl, pure methyl (control), 95% amine and 5% methyl (v/v), and 95% hydroxyl 5% methyl (v/v). Amine SAMs were assembled and rinsed in basic solutions. Hydroxyl SAMs were prepared as backup surfaces to test the FPM derivatization reaction.

2.2 Surface Characterization Methods

2.2.1 Electron spectroscopy for chemical analysis (ESCA)

ESCA spectra were acquired on a Surface Science Instruments S-Probe instrument equipped with a monochromatized aluminum Ka x-ray source, and a hemispherical analyzer. A pass energy of 150 eV was used to acquire all survey and detail scans for compositional analysis. High resolution C 1s, S 2p and Au 4f spectra were acquired at a 50 eV pass energy. Data analysis was carried out using the Service Physics ESCAVB data reduction software.

Angle dependant ESCA analysis was performed using a variable angle stage at 0, 55 and 80° take-off-angles, where the take-off-angle is defined as the angle between the surface normal and the analyzer lens. All angle data was taken at a pass energy of 150 eV using a 12° slit.

The C/Au ratio was calculated for each surface and used as an estimate of the relative layer thickness. This estimate is based on previous work in our labs from a study of the C/Au ratio as a function of chain length [6]. A plot of these
2.2.2 Time of Flight Surface Ion Mass Spectrometry (TOF-SIMS)

TOF-SIMS spectra were acquired on a Physical Electronics PHI 7200 time-of-flight spectrometer using an 8-keV Cs+ ion source in the pulsed mode. The ion source was operated with a pulse width of 1.2 ns and a current of 1.5 pA. Secondary ions of a given polarity were extracted into a two-stage reflectron TOF mass analyzer and detected using a pair of stacked micro channel plates equipped with a time to digital converter. Spectra were acquired using an analysis area of 0.01 mm² and the total ion dose was maintained below 2 x 10¹² ions/cm². Spectra were acquired for both positive and negative secondary ions over a mass range of m/z = 0 to 1000. Negative spectra were calibrated using the CH-, C₂H-, and AuS- peaks. Positive spectra were calibrated using the CH₃+, C₂H₅+, and AuSCH₂+ peaks. Calibration errors were below 10 ppm. Mass resolution (M/DM) for a typical spectra was between 5000 and 6000.

2.3 Derivatization

To test the availability of the NH₂ groups for derivatization, the surfaces were reacted with pentafluorobenzaldehyde (PFB). Derivatization was performed in a large, clean, dry test tube, capped with a stopper wrapped in Teflon tape. A small amount of PFB (Aldrich, 98%) was placed inside the chamber and heated to ~45-55°C. The samples were then placed on a glass slide and put in the reaction chamber for 3 h. The samples were then rinsed with ethanol and dried with nitrogen gas. Fluorescence analysis was performed using a Perkin Elmer Luminescence Spectrometer LS50B.

3. RESULTS AND DISCUSSION

Results of SAM Set I are listed in Table I. As seen in the table, the C10 surface had a high amount of oxygen and a lower C/Au ratio than expected. The lower C/Au ratio and presence of oxygen suggest that these layers were disordered and still contained adventitious contaminants. The amine and mixed surfaces also showed a high percentage of oxygen. It is also noted that the C/Au ratio for the pure amine surface was higher than expected (2.28 vs. ~1.9 expected). The C/Au ratio for the mixed amine/methyl was lower than expected, suggesting a disordered surface. The higher C/Au ratio for the pure amine surface may be due to the presence of physisorbed thiols that were not rinsed off.
or other contaminants. The presence of physisorbed thiols may be supported by the high levels of oxidized sulfur seen on the pure amine surfaces (data not shown).

It is also possible that the amine nitrogens have been oxidized or reacted with species like CO₂ in the air. Also, if the amine nitrogen was in a protonated state, it would carry a positive charge which could interact with polar molecules such as water. Similar considerations have been discussed in a study by Hooper et al., where they also saw the presence of unexplained oxygen on their amine surfaces [7].

Table I also shows that the mixed amine/methyl SAM has only a small amount of nitrogen on the surface. This suggests that the methyl thiol had a higher driving force for the surface than the amine during the assembly.

Table II shows the ESCA results from SAM Set II. As seen in the table, the samples rinsed in basic solution showed lower percent oxygen. These surfaces also showed no oxidized sulfur in the sulfur detail scan (data not shown). Samples assembled in base or neutral solution contained the highest percent nitrogen. Most all surfaces had C/Au ratios higher than expected and still showed significant oxygen contamination.

From this data, we determined the current optimum conditions for assembling amine SAMs to be basic assembly and basic rinsing. This conclusion was based on the lower percent oxygen on the basic rinsed samples and the absence of oxidized sulfur.

Table III lists the ESCA results for SAM Set III. The data shows that in mixtures of 70% amine and 30% methyl for either 1 h or 2 days, there is no nitrogen present on the surface. The mixtures of 90% amine 10% methyl show nitrogen percentages of 2.5 and 2.3 respectively, corresponding to approximately 30% amine on the surface. Therefore, much higher solution percentages of amine thiol must be used to get more amine on the surface.

Angle Dependent ESCA analysis was performed on Sample Set III in order to determine the arrangement of the thiols on the surface. Table IV shows the results of this analysis. As expected, the sulfur percentage decreases going from an angle of 0° to 80° (sampling depth of approximately 60 and 15Å respectively). This indicates that the sulfur is located at the gold surface as expected. Also the nitrogen and oxygen percentages increase from 0° to 80°, indicating that they are on the outside of the layer opposite of the gold surface. This verifies that the amine thiols are not assembling upside down, which could be possible due to the slight affinity of amines for gold. Since the oxygen is located at the outer surface and no oxidized sulfur was detected, the oxygen contamination must be interacting with the top of the layer. This could mean the nitrogen has been oxidized or is interacting with some type of oxygen containing contaminant.

Table V lists the ESCA results for the derivatization with PBF. The PBF should react with all free amines on the surface. Therefore, a relative percentage of reacted amines can be inferred from the fluorine percentage on the surface. The table shows fluorine percentages of 0.9 - 3.4.
Since the PBF would add five fluorines for every nitrogen that is reacted, the fluorine percentage must be divided by five to find the percentage of PBF on the surface. It is noted that the plain gold surface showed 0.9 percent fluorine. This may suggest that some of the fluorine seen on the other surfaces is simply physisorbed and not reacted. The 90/10 sample has a fluorine percentage of 1.7 (0.34 % PFB). When this is compared to the total nitrogen percentage, it shows that at best only 11.0 % of the nitrogens on the surface reacted. This supports the possibility that the nitrogens are somehow complexed with some compound such as water or they have oxidized or reacted and are not available for derivatization. Finally, the fluorine present on the gold control and 70/30 sample are indicative that the reaction conditions were most likely not fully optimized (due to time constraints, optimization was not possible).

Samples in SAM Set IV were assembled for reaction with the porphyrin. ESCA analysis was performed to verify the samples had assembled properly (see Table VI). The derivatization reaction can occur with either an amine or hydroxyl group on a SAM. Due to the difficulties encountered with the amine thiols, hydroxyl SAMs were added for comparison with the amine SAMs. As shown in the table, all the samples have potential reactive sites for the porphyrin to be coupled with (O for OH SAMs, N for Amine).

The results from the porphyrin reaction and the fluorescence detection are pending.

4. CONCLUSION

This study proved to be very useful for the characterization of amine SAMs and to begin to determine the best conditions for their assembly. The amine thiols were difficult to work with, but it was shown that with a basic assembly and a basic rinse, the oxygen contamination is greatly reduced and the sulfur oxidation eliminated. Further studies might show that the oxygen contamination could be eliminated through increased basic conditions or other methods. The oxygen contamination may be due to water interacting with the protonated amine, or the amine oxidizing or reacting. High resolution ESCA analysis of the nitrogen should be able to determine the bonding state of the amine. This may help determine the source of the contaminating oxygen and determine whether the nitrogen has reacted and/or oxidized. The results of the porphyrin reaction and fluorescence detection are pending. Hopefully these results will help determine the suitability of using SAMs in DNA microarray technology.

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REFERENCES

An Alginate-based Scaffold Containing Amino Acid Networks Displays Improved Biocompatibility In vivo

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Abstract: Implanted biomaterials elicit a chronic inflammatory response that leads to their encapsulation by a poorly vascularized collagenous capsule. This is known as the foreign body response (FBR) and can lead to implant failure. In an attempt to improve biocompatibility, biomaterials that have a reduced inflammatory response are desired. Towards this aim, porous biomaterials consisting of a single polymer and cross-linked with amino acids have been generated. Specifically, alginic acid or PVA was cross-linked with glycine and lysine and made porous (10-50 m$m$m pore diameter). In the present study, the biocompatibility of this material was tested following subcutaneous implantation in mice for four weeks. The implants were excised en bloc, fixed in formalin and embedded in paraffin. Histological and immunohistochemical analysis of sections indicated significantly reduced capsule thickness in comparison to a similarly cross-linked biomaterial made of polyvinyl alcohol. Within the interstices of the alginate scaffold, we observed the presence of foreign body giant cells, numerous blood vessels, and the deposition of mature collagen fibers. On the contrary, PVA-based scaffolds elicited a pronounced chronic inflammatory response. In conclusion, this modified alginate biomaterial elicits a reduced FBR and promotes formation of blood vessels and matrix deposition. These characteristics make this biomaterial an excellent candidate for tissue engineering applications.

1. INTRODUCTION

Implanted biomaterials, due to their size and surface chemistry, elicit a response from the host that aims to isolate the biomaterial from the surrounding tissues. This is known as the foreign body response (FBR) and is non-specific protection mechanism. However, this reaction poses a problem for most implants since the FBR surrounds them with a collagenous capsule that is largely avascular. Thus, it isolates the implant and depending on the type of implant it can render it non-functional [1].

Attempts to improve the biocompatibility of biomaterials have focused on decreasing non-specific protein interactions, reducing capsule thickness, and by allowing them to degrade over time [2]. In tissue engineering applications, it is necessary to have biomaterials that direct surrounding cells to grow and migrate in specific 3D patterns. Such scaffolds are formed from polymers that cross-link into an open network that allows cells to infiltrate and adhere [3]. In this study, we attempt to characterize such a network formed from two different polymers - alginic acid and polyvinyl alcohol (PVA).

2. MATERIALS AND METHODS

2.1. Antibodies, Reagents, Biomaterials

Polyclonal anti-PECAM-1 antibody was purchased from Pharmingen. Immunohistochemistry Vectastain ABC kit was purchased from Vector Laboratories. Alginic acid and polyvinyl alcohol was cross-linked with glycine and lysine to form porous sponges (Nair et al., manuscript in preparation). All sections were examined with the aid of a Nikon Eclipse 800 microscope and the digital images were captured with the aid of a Photometric digital camera. The Metamorph® program (Version 4.1) from Universal Imaging Corporation® was used to quantify angiogenesis.

2.2 Implantation of Modified PVA and Alginate Sponges

Three polyvinyl alcohol (PVA) sponge samples and three alginate sponge samples implanted in three mice were analyzed in this study. Biomaterials were implanted in wild-type mice as described previously [1].

2.3 Tissue Processing – Analysis of the FBR

The sponges were excised 4 weeks following implantation, fixed in zinc-buffered 10% formalin, and embedded in paraffin. Paraffin sections were stained with the Masson’s Trichrome, and with the antibody to PECAM-1, as described previously [4]. Prior to incubation with the primary PECAM-1 antibody, the sections were treated with 1:1 weight ratio of .25% trypsin and .025% pronase for 10 min. at 37°C.

2.4 Capsule Quantification

Relative thickness of the FBR capsule was measured with the aid of an ocular micrometer. Conversion factors were obtained from a stage micrometer and used to calculate absolute capsule thickness in microns.
2.5 Vessel Quantification

The area for each blood vessel was accurately measured by outlining figures that stained positively for the platelet endothelial cell adhesion molecule, PECAM-1, as described previously [5].

3. RESULTS

3.1 Histological Analysis of the FBR

Figure 1 shows a representative image of a section stained with Masson’s Trichrome stain, which displays a high degree of cellular invasion in the alginate-based biomaterial. The blue areas of the stain indicate mature collagen fibers that formed within the interstices of the scaffold and are the main component of the FBR capsule. Also visible are large multi-nucleated cells, known as the foreign body giant cells (FBGC). These cells form by fusion of macrophages, a process known as frustrated phagocytosis. In addition, blood vessels of various sizes are observed within the interstices of the alginate scaffold. Figure 2 shows representative images from alginate (A) and PVA (B) implants. The capsule surrounding the alginate scaffold was reduced in thickness and was largely avascular. On the contrary, the PVA scaffolds elicited robust encapsulation and inflammation. The latter may be responsible for the increase in capsule vascularization in these implants.

3.2 Quantification of the FBR Capsule

The thickness of the capsule around the implant can be considered as a measure of the intensity of the FBR. A thicker capsule indicates a greater isolation of the sponge from the surrounding tissue, and a thinner capsule suggests better biocompatibility. The capsule elicited by the alginate scaffold (51.4 ± 33.0 µm) is significantly thinner than that of PVA (199.4 ± 86.1 µm) (Fig. 2C).

3.3 Quantification of the Angiogenic FBR in Alginate-Based Biomaterials

Blood vessels were observed within the alginate sponge (Fig. 2A). Three implanted samples were analyzed, with 33, 29, and 30 high-powered digital images taken from each sample. The average vessel size (lumen area) in the alginate scaffold was 195.4 ± 38.9 µm². This represents a significant increase over the vessel size that was previously determined in PVA sponges (108.6 ± 10.0 µm²) (Fig. 3).
This study indicates that various types of cells are successful in migrating, proliferating, and remodeling ECM in the alginate-based biomaterial. In addition, this biomaterial elicits a minimal FBR, suggesting that it may be suitable for tissue engineering applications.

The increased capsule thickness surrounding PVA scaffolds is surprising. PVA sponges and other biomaterials (polydimethylsiloxane, millipore filters) elicited FBRs that were less robust than those obtained for the PVA scaffolds in this study [1, 2]. This suggests that the FBR is further intensified after cross-linking PVA to amino acids, and is therefore not conducive towards generating a better biomaterial.

There is increased biodegradation of the alginic acid network than of the PVA network, which might be correlated with the bigger blood vessels in the alginate scaffold. As the biomaterial disintegrates, it creates space for more cells to infiltrate and for the pre-existing blood vessel lumens to grow larger. In addition, the possibility that the size of blood vessels can be influenced by the degradation products cannot be excluded. To confirm this hypothesis, a method needs to be developed that would quantify the change in area occupied by a biomaterial, and correlate that change with the dimensions of infiltrating blood vessels.

In future studies of this technique for generating better biomaterials, it would be beneficial to compare sponges with similar pore sizes. That would allow us to differentiate the effects of porosity from the effects of additional cross-linkages on the FBR. Further modifications to develop more biocompatible material could involve combining the alginate material presented here with DNA delivery as part of a scaffold. It has been shown that coating biomaterials with anti-sense thrombospondin 2 DNA can modulate the FBR and increase angiogenesis [2]. By using that in conjunction with the modified biomaterial, it would be possible to increase the access of cells to nutrients and promote their growth on a scaffold. In conclusion, cross-linking alginic acid with amino acids results in a better biomaterial that elicits a reduced inflammatory response. It may be used to direct three-dimensional cell growth for various tissue-engineering applications.

**REFERENCES**

Skeletal Myoblast Motility on Laminin Patterned Surfaces

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Abstract: Terminally differentiated skeletal muscle cells are long “syncytia” (multinucleated cells) formed by the fusion of many precursor cells, called myoblasts. Therefore, understanding how myoblasts attach, migrate, divide, and fuse on biomaterials is critical to developing functional engineered skeletal muscle tissue. This study uses a permanent myoblast cell line (MM14) derived from adult mouse skeletal muscle. With the aid of video microscopy and imaging software, myoblast motility was examined on varying concentrations of the extracellular matrix protein, laminin. To accomplish this, the cells were spatially restricted using micropatterning techniques. The two objectives in this study were (a) to determine the laminin concentration(s) that permit the fastest cell migration; and (b) to determine whether myoblast cell motility changes over time in culture.

1. INTRODUCTION

Skeletal muscle, such as that in the arm or leg of an adult, consists mostly of mature, multinucleated muscle fibers. These terminally differentiated cells are post-mitotic (i.e., they no longer divide). However, there is also a small population of quiescent muscle cell precursors, known as satellite cells, located anatomically at the periphery of the mature muscle fibers. The function of satellite cells is to re-enter the cell cycle in response to skeletal muscle trauma, and to produce a pool of myoblasts, which can fuse to replace the injured muscle. However, even with this endogenous repair feature, skeletal muscle is incapable of complete functional restoration after massive trauma.

The long-term goal of our study is to improve healing of severely damaged muscle tissue. One approach is the use of engineered skeletal muscle tissue grafts [1]. A proposed scheme of muscle cell replacement therapy is to use skeletal myoblasts (which are replicative cells; see above) to populate a biomaterial, which facilitates parallel cell align-
The implant could be synthesized and coated with cell adhesion matrices, hormones, signal molecules, or growth factors. Myoblasts prefer specific extracellular proteins for attachment, division, and differentiation; thus, the biomaterial will need to be coated with one or more of these biological components [2]. Coating flat surfaces can be done with micropatterning [3]. In this study, we used one of these preferred extracellular proteins, laminin, and patterned it on a flat polystyrene flask surface. Several laminin isoforms have been identified, including laminin-1, laminin-2, and laminin-5, etc. These proteins are localized to the basal membrane, which surrounds muscle fibers in vivo. Laminin contains three subunits (∝, ∞, and γ). The a subunit binds directly to cell surface receptors known as integrins (Fig. 2) [4]. Integrins are transmembrane receptors, consisting of an ∝ and ∞ subunit, whose ligands are basal membrane proteins. The integrins, along with its ligand, serve a variety of paired functions: from cell motility to cytoskeletal arrangement [4]. The laminin used in this study is laminin-1.

Muscle in the body naturally differentiates as unbranched, parallel, and highly organized muscle fibers. In tissue culture, myoblasts are cued to undergo differentiation by switching from a medium containing high growth factor levels to one lacking special growth factors. When skeletal muscle myoblasts are allowed to migrate and differentiate on unpatterned laminin surfaces they form highly branched and non-parallel myotubes, which does not mimic natural muscle [5]. We predict, therefore, that skeletal myoblasts must first differentiate into long unbranched, parallel myotubes in order to be functional grafts. The use of pattern surfaces allows us to mimic this in vivo arrangement. When skeletal myoblasts are induced to differentiate on linear patterned protein, they form long, unbranched and well-organized myotubes [5]. Myotubes in this form are closer in structure to myotubes in vivo, than are the highly branched myotubes created on unpatterned protein.

The process of creating a patterned protein surface is called micropatterning [3]. Elastomer stamps are molded with a desired patterned on their surfaces. Protein is allowed to absorb onto the stamp and is then transferred to a plastic culture surface.

We examined the migration of MM14 cells, a mouse skeletal muscle myoblast cell line, on micropatterned surfaces of laminin-1. The two goals of the study were to determine which laminin concentration resulted in the fastest cell migration and if the cell velocity of undifferentiated cells changed over time in vitro.

2. MATERIALS AND METHODS

2.1 Stamping Preparation
The stamping wafers, or molds, were produced from a master template containing various lane widths and lane separations, anywhere from 5-45µm lane widths and up to 80µm separation distances. 5µm x 80µm (lane width x lane separation) silicone elastomer stamps were molded using a 10:1 mixture of elastomer: curing agent (Sylgard 184, Midland Mich.). The stamps were coated for 30 min. with the following laminin concentrations: 15, 45, or 135mg/ml (Sigma, St. Louis MO). The stamps were then rinsed with phosphate buffered saline (PBS), distilled water (diH2O), and dried with filtered air. Stamps were used immediately after drying (Fig. 3).

2.2 Stamping Surface
T25 tissue culture flask surfaces were blocked with 10ml of 1% bovine serum albumen (BSA) for 1 h at 4°C, then rinsed three times each with PBS and diH2O. The stamping surface was dried completely with filtered air. The stamps were then placed laminin side down inside the flask and allowed to sit from 2 to 5 min. After removing the stamp, the surface was covered with PBS and stored at 4°C until needed.

2.3 Cell Culture
Mass cultures of MM14 mouse myoblasts were maintained in F10C media supplemented with 15 % horse serum (HS) in a 37°C incubator with 5% CO2. In order to keep the cells proliferating 4 ng/ml fiberblast growth factor (FGF) was added every 12 h. To establish “patterned

**Figure 3**
Stamping of Laminin micropatterns. (A) Solutions of known laminin concentrations were pipetted onto polymer stamps and allowed to adsorb. (B) The stamps were rinsed, and the rinsed stamps were printed on BSA-blocked culture flasks. (C) After 2-5 min., the stamps were lifted off, creating a laminin-patterned surface (horizontal lines).
cultures,” cells were trypsinized and plated onto stamped flasks, which had been pre-equilibrated with 6.5 ml media and 4 ng/ml FGF. After allowing the cells to attach for 45-60 min, unattached cells were removed by aspiration and the cultures were re-fed. The cultures were then equilibrated with 5% CO₂ to regulate the pH, and the flasks were sealed.

2.4 Time-Lapse Video Microscopy
The cells were maintained at 37°C during time-lapse video capturing. Time-lapse images were collected at 30 sec intervals for 1 h using a Nikon TE200 inverted microscope with cooled digital camera. One field was chosen and photographed for 3 h.

2.5 Immunostaining
After data collection the cells were fixed in alcohol/formaldehyde/acetic acid (AFA). After fixing, the cells were immunostained with an antibody specific for sarcomeric myosin (MF20), and then counter stained with Hematoxylin.

2.6 Data Processing and Analysis
MetaMorph imaging software (Universal Imaging Corporation) was used to gather data on cells in the areas photographed [7]. The algorithm chosen for the program used a gray-scale threshold difference to locate a cell’s position. The program was given gray-scale contrast standards for each cell tracked and a 50% range around the threshold to “search” for the cell in the succeeding frame (Fig. 4).

3. RESULTS
We were able to effectively spatially restrict skeletal myoblasts to the patterned laminin (Fig. 5). The Figure 5 image is one frame out of 121 taken over 1 h and contains approximately 90 cells. The frames were then compiled into a movie (note the parallel patterns on which the cells travel). Figure 6 illustrates how far a particular cell moved after 1 h, or 121 frames. The white arrow is the cell’s position at time-1 and the black arrow is the position at time-2 (60 min. later). In this 1-h demonstration the cell moved 90µm only to the left. However, other cells migrated only to the right, and some moved in both directions.

It is clear that the cells move on the laminin patterns. Videotape data were collected commencing 1 h after the cells were plated. The average cell velocity was calculated for 3 consecutive hours by tracking individual cells using the movies created with the MetaMorph software. The question, does the cell’s velocity decrease or increase with time, was raised while recording the time-lapse videos. Cells were chosen from the 135µg/ml data and analyzed over a 24-h period. The cells showed no statistical increase or decrease in velocity. Of note, the cells were slightly slower at hour one, perhaps due to plating and transferring to the filming location (Fig. 8).

After statistical tests were completed (using ANOVA) it
was found that there was no statistical difference in velocity among the three concentrations of laminin tested (15, 45, and 135 µg/ml) for 1, 2, 3 h (see Discussion). These data are shown in Table I, Figure 7, and Figure 8. Table 1 lists the cells’ mean-velocities for each time period of capture.

## 4. DISCUSSION

Like laminin, fibronectin is a basement membrane protein involved in cell migration. Previous studies with patterned fibronectin showed that cell velocity was proportional to protein concentration, and reached a max at an intermediate concentration and then declined [6]. In the interest of developing an optimal artificial graft for skeletal muscle therapy, we were interested in whether myoblast migration on laminin was also “biphasic.” Previous studies in our lab showed cell velocity was greater at 45 µg/ml applied laminin than 4.5 µg/ml [5]. This study extended these observations by examining laminin concentrations greater and less than 45µg/ml.

Results presented here suggest cell velocity increases between 4.5 and 15 µg/ml laminin and then remains relatively constant up to 135 µg/ml. However, more experiments are needed in order to show this unequivocally. We also demonstrate that cell velocity remains constant for at least 24 h. The persistence of myoblast migration can be advantageous in populating a complex and porous biomaterial. Another advantage from an engineering perspective is that it might be possible to control cell motility by culturing cells on surfaces containing different laminin concentrations.

However, laminin concentrations may also affect other aspects of cell behavior such as attachment and perhaps more importantly, the “quality” of muscle differentiation. Studies addressing these behaviors are in progress. Other studies include: determining how much laminin is actually being transferred during stamping and the effect of myoblast differentiation on migration velocity.

### Table I

Experimental Data. Velocity, laminin concentration, and statistics.

<table>
<thead>
<tr>
<th>Time Interval of data acquisition (hr)</th>
<th>Laminin Stamping Concentration (µg/ml)</th>
<th>Average Number of cells analyzed / experiment</th>
<th>Number of Experiments</th>
<th>Average Velocity (30 sec intervals, µm/min)</th>
<th>Standard Error of the Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>23</td>
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<td>2.40</td>
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<td>135</td>
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<td>1</td>
<td>2.82</td>
<td>.13134</td>
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</tbody>
</table>
FIGURE 7

The effect of laminin stamp application concentration on myoblast cell velocity. Data collection started approximately 2 h after the cells were plated. Where indicated, the data are grouped within the 1st, 2nd, and 3rd hour of data collection. Previous data (Year 2000) indicated that cells moved faster on 45 $\mu$g/ml laminin than 4.5 $\mu$g/ml (5). A statistical test (ANOVA) indicated no statically significant difference in cell velocity on laminin concentrations: 15 $\mu$g/ml, 45 $\mu$g/ml, 135 $\mu$g/ml (data shown are means and SEM from pooled experiments with 2, 1, and 3 experiments respectively and 11 to 29 cells analyzed/experiment).

FIGURE 8

Myoblast cell velocity remains constant over time on laminin micropatterns.
AGKNOWLEDGEMENTS

Thanks goes out to Dr. McDevit, Dr. Hauch, the entire Hauschka Lab (special thanks to Dr. John Angello and Dr. Robert Welikson), and to Fanaye and the entire UWEB staff for giving me the opportunity to succeed and excel.

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**Controlling Foreign Body Giant Cell Formation with Osteopontin**

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**Abstract:** One of the defining characteristics of the chronic inflammatory response to implanted biomaterials is the fusion of macrophages to form foreign body giant cells (FBGCs) at the tissue-biomaterial interface. Since FBGCs have been implicated in biomaterial degradation and tissue injury, efforts have been made to control their formation. Recently, the protein osteopontin (OPN) has been shown to reduce FBGC formation in vitro. In order to develop OPN as a therapeutic coating on biomaterials, we would like to determine how OPN exerts its effect. We hypothesize that the arginine-glycine-aspartate (RGD) amino acid sequence of OPN interacts with the αvβ3 integrin on macrophages to inhibit their fusion. In order to evaluate whether the RGD sequence of OPN is critical to its function, three different proteins were produced. These proteins were then used in an *in vitro* FBGC assay to examine their influence on FBGC formation. Due to sub-optimal protein production techniques and contamination during the FBGC assay, the data collected is insufficient to determine whether the RGD sequence of OPN plays a critical role in FBGC formation.

**1. INTRODUCTION**

The foreign body reaction is the typical immune response to an implanted synthetic material [2, 22]. Once a biomaterial is implanted into the body, it adsorbs a layer of non-specific proteins that serve as scaffolding for monocytes/macrophages to adhere to and “attack” the biomaterial. When the macrophages find that they cannot digest the implant, they fuse into larger multinucleated foreign body giant cells (FBGCs). However, due to the overbearing size of the biomaterial, FBGCs are also unsuccessful at engulfing the implant. Nonetheless, the FBGCs typically remain at the surface for the lifetime of the device and secrete degradative enzymes and reactive oxygen intermediates, an activity coined “frustrated phagocytosis” [2].

The presence of foreign body giant cells at the surface of an implant has been implicated in undesirable biomaterial degradation [1]. Further, the products secreted by FBGCs may damage surrounding tissue, and the constant low-level activation of FBGCs and macrophages may cause systemic immune-weakening effects. Finally, FBGCs send out cytokines that attract fibroblasts to the implant site. The fibroblasts synthesize a collagenous capsule around the implant, effectively walling off the implant from the rest of the body. Encapsulation inhibits the performance of implanted devices by preventing direct contact between the device and the tissue [22].

For the above reasons, it is desirable to be able to control the formation of FBGCs. In our lab, it has been shown that the protein osteopontin (OPN) may play a role in mediating FBGC formation. *In vivo*, OPN knockout mice show increased FBGC formation and *in vitro*, soluble OPN reduces FBGC formation in a dose-dependent manner. [unpublished results]. In order to gain more insight into these results and eventually develop OPN as a therapeutic coating on biomaterials, we would like to determine how OPN exerts its effect. We hypothesize that the arginine-glycine-aspartate (RGD) amino acid sequence of OPN interacts with the αvβ3 integrin on macrophages to inhibit their fusion. In order to evaluate whether the RGD sequence of OPN is critical to its function, three different proteins were produced. These proteins were then used in an *in vitro* FBGC assay to examine their influence on FBGC formation. Due to sub-optimal protein production techniques and contamination during the FBGC assay, the data collected is insufficient to determine whether the RGD sequence of OPN plays a critical role in FBGC formation.

**FIGURE 1**

Schematic diagram of human native osteopontin and the recombinant human osteopontin fragments used in the *in vitro* FBGC assay. Numbers indicate the amino acid number in reference to human osteopontin. The preparation of these proteins is described in the “experimental procedures” section. This figure has been adapted from Smith 1999 and Sodek et al., 2000.
osteopontin exerts its effect. Initial experiments in vitro have revealed that osteopontin does NOT reduce occurrences of giant cells by causing death of the giant cells. Further, in this assay, macrophages are immobilized on the surface before FBGCs formation is invoked (see Experimental Procedures). Therefore, OPN must inhibit the formation of giant cells by inhibiting the fusion of monocytes/macrophages, the mechanism of which remains to be determined.

Osteopontin is a glycoprotein that serves to modulate many macrophage activities, including macrophage migration to the site of a tissue injury [20, 27]. Some of these activities are known to be caused by an interaction between the arginine-glycine-aspartate (RGD) domain of osteopontin and the $\alpha_v\beta_3$ integrin on macrophages [26, 20]. Furthermore, $\alpha_v\beta_3$ integrin expression on monocytes is required for the formation of multinucleated osteoclasts (osteoclasts and FBGC’s are two different types of giant cells derived from monocytes) [8]. Therefore, it is reasonable to suppose that the $\alpha_v\beta_3$ integrin plays a role in the formation of FBGC’s from macrophages. We hypothesize that the RGD motif of OPN interacts with the $\alpha_v\beta_3$ integrin on macrophages to inhibit the fusion of macrophages.

In order to examine the role of the RGD sequence in preventing FBGC formation, three different proteins were produced (Fig. 1): (1) a full-length correlate to human OPN that can be expressed recombinantly in bacterial cells; (2) the N terminal portion of human osteopontin containing an RGD sequence that results from thrombin cleavage of full-length osteopontin (this protein is designated as 30N-OPN RGD); (3) the same N terminal fragment except that the RGD sequence has been mutated to RGE. This protein is designated as 30N-OPN RGE (see Fig. 1). The effects of each protein on FBGC formation were compared in vitro.

2. EXPERIMENTAL PROCEDURES

2.1 Cell Lines

Bovine aortic endothelial cells (BAEC) were isolated from bovine aortas as described previously [24] and maintained in Waymouth’s MB 752/1 medium (Invitrogen, Inc.) containing 10% fetal bovine serum and 1% penicillin-streptomycin.
Hung-Kam Cheung and Dr. Leona Ling (Biogen), and the GST 30N-OPN RGE construct was created by site directed mutagenesis of OP30 [23] using the QuickChange site-directed mutagenesis kit (Stratagene Cloning Systems, Inc.).

2.3 Protein Analysis

Protein concentrations were determined using the Micro BCA Protein Assay Reagent Kit (Pierce) and BSA protein standards. Absorbance was measured at 560 nm. Qualitative analysis of protein purity was performed with 10 or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). One of the gels was stained with coomassie blue, and the other was immunoblotted with the primary, polyclonal antibody OP189 (described in Smith 1999), and the secondary antibody horseradish peroxidase (HRP), labeled rabbit anti-goat. The functionality of the proteins was evaluated with a cell adhesion assay, as follows. Proteins were coated onto 96-well Maxisorp microtiter plates (Nunc, Inc.) at a concentration of 100 nm and incubated overnight at 4°C. They were subsequently blocked with 10 mg/ml bovine serum albumin (BSA, Fraction V from Sigma) in PBS for 1 h at 37°C under 5% CO₂. The cell mixture was seeded into 24-well untreated polystyrene plates (Becton-Dickson) at a density of 10⁶ cells/ml of RPMI-1640 media, supplemented with L-glutamine (Gibco BRL), 25mM HEPES (Gibco BRL), 1mM MEM sodium pyruvate (Sigma), 0.1 mM MEM non-essential amino acids (Sigma), 1% penicillin-streptomycin (Sigma), and 25% autologous human serum (AHS). BAECs were resuspended in 1mg/ml BSA in Waymouth’s MB 752/1 medium (no serum) (Invitrogen, Inc.) and approximately 50,000 cells were added to each well and incubated for 2 h. Attached cells were stained with toluidine blue and quantified by reading the absorbance at 630 nm. Fibronectin at a concentration of 50 nm was used as a positive control and PBS was used as a negative control.

2.4 Isolation of Human Monocytes

Human peripheral monocytes and lymphocytes were isolated from healthy, medication-restricted adults by density centrifugation (29) with Histopaque-1077 (Sigma). Monocytes were separated from the lymphocytes based upon differences in their ability to adhere to polystyrene, as follows. The monocyte/lymphocyte cell mixture was seeded into 24-well untreated polystyrene plates (Becton-Dickson) at a density of 10⁶ cells/ml of RPMI-1640 media, supplemented with L-glutamine (Gibco BRL), 25mM HEPES (Gibco BRL), 1mM MEM sodium pyruvate (Sigma), 0.1 mM MEM non-essential amino acids (Sigma), 1% penicillin-streptomycin (Sigma), and 25% autologous human serum (AHS). Subsequently, the monocytes were incubated for 2 h at 37°C under 5% CO₂. Finally, the cell mixture was rinsed in serum-free RPMI 1640 (Gibco BRL) to remove non-adherent lymphocytes, then replenished with the supplemented RPMI media.

2.5 Determination of the Effect of Proteins on FBGC Formation

2.5.1 In Vitro FBGC Assay

The effect of the proteins on FBGC formation was tested with an in vitro FBGC assay. Each protein sample was tested in triplicate, and appropriate controls were provided, as shown in Figure 2.

For endotoxin control treatments, monocytes were stimulated with 5 mg/mL lipopolysaccharide (LPS; Sigma) immediately after monocyte isolation to invoke the transition from monocyte to macrophage. FBGC formation was induced in vitro using interleukin-4 (IL-4; Sigma) and granulocyte-macrophage colony stimulating factor (GM-CSF; provided by Immunex, Inc.) in a ten-day assay developed by Anderson and McNally (1995). On days 3 and 7, the media was replaced with heat-inactivated AHS in RPMI, and the following reagents were added: 10 ng/mL IL-4, 10 ng/mL GM-CSF and 500 nM soluble full-length or cleaved osteopontin (filter-sterilized). On day 10, cells were fixed with methanol, air dried and stained with May-Grünwald and Giemsa reagents (Sigma).

2.5.2 Evaluation of FBGC Formation

Multinucleated FBGC formation was evaluated visually by brightfield microscopy. The number of macrophages and FBGC’s (including their number of nuclei) in three representative fields of view (~0.25 mm² each) for each well were summed and then averaged over three wells (per treatment).
3. RESULTS

3.1 Protein Analysis

3.1.1 His-OPN and 30N-OPN RGD are Suitably Pure but 30N-OPN RGE is Not

In order to determine the purity of the protein preparations, SDS-PAGE analysis was performed. Results from the analysis of each protein are shown below. The coomassie dye stains ALL proteins while the western blot stains only proteins related to osteopontin.

Figure 3 shows the SDS-PAGE analysis of His-OPN. For two reasons, the major band appearing at 50kDa in the coomassie stain is most likely His-OPN even though the actual molecular weight of His-OPN is 34kDa. First, this band is also very heavy on the western. Second, the adhesion assay shows that the major protein produced from the preparation supports high levels of cellular adhesion (Fig. 6). The discrepancy between the apparent molecular mass and the actual molecular mass is likely due to charge effects. Since this main band in the coomassie also appears in the western, the His-OPN is suitably pure. The second darkest band appearing at just under 25kDa may be a degradation product of the osteopontin.

Figure 4 shows the SDS-PAGE analysis of the 30N-OPN RGD fragment. Note that the coomassie stain was performed on a 10% resolving gel, while the western blot was performed on a 12.5% resolving gel. Therefore, it is likely that the major band in the coomassie corresponds to the major band in the western blot. Since the actual molecular weight of the 30N fragment is 17kDa, it is also likely that this band is consistent with the desired fragment, in which case the 30N-OPN RGD is acceptably pure.

Figure 5 shows the SDS-PAGE analysis of the 30N-OPN RGE fragment. It does not appear that the main band from the coomassie stain corresponds to any band from the western blot. Furthermore, the main band in the coomassie stain appears at a molecular weight of close to 30kDa, which is much different from the actual molecular weight of the 30N fragment (17kDa). However, the dark band in the western blot around 23kDa may correspond to the RGE fragment. If this is the case, then it is clear that the 30N-OPN RGE produced is very impure. However, it may also be the case that the polyclonal antibody used for the western blot does not effectively recognize the 30N-OPN RGE fragment, so it would not show up on the western blot regardless of its concentration.

3.1.2 All Three Proteins are Functional

In order to determine whether the proteins produced are functional, an adhesion assay was performed using BAEC cells. As shown in Figure 6, all three of the OPN proteins produced appear to be functional. His-OPN and 30N-OPN RGD support levels of adhesion near the positive control. As expected, the mutated 30N fragment (30N-OPN RGE) supports less cell adhesion than the native forms of OPN (but more than the negative control) because a major cellular attachment site (RGD) has been mutated (to RGE).

3.1.3 All Three Proteins were Produced In Sufficient Quantity

In order to quantify the amount of protein obtained from each protein preparation, a BCA assay was performed. A summary of these results is shown in Table 1. For the foreign body giant cell assay, proteins were added at a concentration of 500 nm and the total volume of media per well was 1.0 mL. Therefore, each well requires 8.5 µg of protein for the fragments (MW 17,000), or 17.5 µg of protein for the His-OPN (MW 34,000). The total amounts of the proteins produced allow for at least three wells of each protein treatment, as required.

3.2 Effect of Proteins on FBGC Formation

3.2.1 None of the Three Proteins Reduced Foreign Body Giant Cell Formation

To quantify differences in FBGC formation due to the different protein treatments, the percentage of nuclei within giant cells was tabulated. Figure 7 displays the distribution of nuclei after completion of the 10-day FBGC assay. As expected, there are a greater percentage of nuclei within giant cells with
the positive control (IL-4, GM-CSF, no protein) than with the negative control (monocytes only). However, neither protein reduced FBGC formation to a significant extent compared to the positive control. This is true at all sizes of giant cells in the range from \( \geq 2 \) nuclei to \( \geq 6 \) nuclei. Furthermore, there are no distinguishable differences between the different proteins. The endotoxin control (LPS + IL-4 + GM-CSF) visually seems to have reduced FBGC formation somewhat, so any decrease in FBGC formation that may have been seen with the protein treatments may have only been due to contamination of the protein sample with LPS.

3.2.2 Cell Survival is not Uniform Among Treatments

Total numbers of nuclei were tabulated to indicate whether any treatments are promoting cell death/inhibiting cell survival. As Figure 8 shows, treatments involving LPS or His-OPN show substantially lower survival rates than the other treatments, indicating that these treatments may be killing the cells.

4. DISCUSSION

4.1 Protein Analysis

Based upon the protein analysis results, it appears that the His-OPN and the 30N-OPN RGD fragment were produced in sufficient quantity and with sufficient purity and functionality. However, the 30N-OPN RGE fragment is of questionable purity. Therefore, any results observed in the FBGC assay must be verified by repeat experiments, particularly involving the 30N-OPN RGE fragment.

4.2 Effect of the Proteins on FBGC formation

The results of this FBGC assay contrast starkly with previous work. In previous FBGC assays performed in our lab, 500nM His-OPN reduced FBGC (\( \geq 3 \) nuclei) formation to a significant extent (\( p = 0.01 \)) compared to the positive control. Also, fusion rates were generally much higher [1]. Furthermore, cell survival was uniform across all treatments, suggesting that the OPN inhibits FBGC formation by preventing the fusion of cells and not by causing death of the giant cells. Finally, cell density within the wells was visibly higher in past assays.

There are several possible explanations for the discrepancy between the results seen here and the results previously seen in our lab. First, the LPS and His-OPN aliquots may have been contaminated, leading to the low cell survival levels seen. There could also have been widespread (over all wells) contamination incurred during the 10-day FBGC assay. Lastly, the protein preparations may not have been pure enough.

In conclusion, with the data accumulated, it is not sufficiently clear whether OPN’s effect on FBGC formation is RGD-mediated. In order to conduct future trials successfully, the protein production process must be optimized (par-

### Table I

A summary of the quantitative analysis of the protein preparations. “Concentration” and “total volume” refers to the protein/PBS solution obtained after purification using the techniques described in the “Experimental Procedures” section. The concentrations were determined by BCA assay. The 30N-OPN RGD fragment was produced using a total of 3L of LB broth. The 30N-OPN RGE fragment was produced using a total of 2L of LB broth and the full length His-OPN was produced using 1L of LB broth. The final volumes of the protein solutions (in PBS) and the total amount of each protein produced are approximate.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (mg/mL)</th>
<th>Approx. total volume (mL)</th>
<th>Approx. total amount (mg)</th>
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</thead>
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</tr>
<tr>
<td>30N-OPN RGD</td>
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</tr>
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<td>Full-length His-OPN</td>
<td>1443.9</td>
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</tr>
</tbody>
</table>
particularly for the 30N-OPN RGE fragment), and the reagents used in the FBGC assay should be rechecked for sterility and purity.

Although the results obtained here are inconclusive, a brief and simplified case analysis will be presented (Table II).

Case 1 would support our hypothesis that the RGD sequence is the functional portion of OPN. Case 3 would neither support nor rule out our hypothesis, and Case 2 would strongly discount our hypothesis. If we had seen RGD dependence (Case 1), it is likely (as mentioned in the introduction) that the integrin $\alpha_v\beta_3$ is the corresponding receptor for the RGD sequence on macrophages. However, considering recent results from McNally and Anderson (2002), other integrin receptors (expressed by macrophages and known to interact with OPN) are possible, including $\alpha_v\beta_3$, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ [7, 20]. If we had seen case 1 or 3, this would lend credence to work implicating non-integrin receptors participating in macrophage fusion, including CD44 [28] and the macrophage mannose receptor [9, 18].

There are several other areas of research that we would like to pursue to extend the ideas presented here. In subsequent assays we would like to evaluate the effect of other mutants of OPN (for example, the 10C fragment) [25]. It may also be beneficial to determine whether other RGD containing proteins have similar effects on FBGC formation (for example, fibronectin [14, 16] and vitronectin). Moreover, since the FBGC assay used in this research only extends for ten days, it may be wise to test whether OPN’s effect extends past the 10-day period. Finally, we would like to determine whether OPN (or a functional fragment of OPN) could be immobilized on the bottoms of the wells and still impede giant cell formation. Ultimately, OPN may be used to modify the surfaces of biomaterials and control FBGC formation in vivo.

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A Novel Way to Control Microcellular Foam Density Using Desorption Time

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Abstract: This study investigates the effect of desorption time on foam density in the batch process for acrylonitrile-butadiene-styrene (ABS), crystalline polyethylene terephthalate (CPET), and polycarbonate (PC). ABS, CPET, and PC were foamed using the batch microcellular process. They were pressurized at 5MPa and foamed at appropriate foaming temperatures at different lengths of desorption time. Results showed that the relative density of ABS increased from 30% to 90% during the first six days of desorption, while CPET went from 60% to 90% in the first day, and the relative density of PC increased from 67% to 99% in the first 20 h of desorption. This study demonstrates that the desorption time can be used as a variable to control foam density.

1. INTRODUCTION

Microcellular polymeric foams are characterized by bubbles with diameters ranging from 10 to 100 µm. Microcellular foams have potential to be used as biomaterials. A review of literature in biomaterials research has shown that porous materials can be used for cell seeding and cell transplantation [1-4]. The ultimate goal is to generate microcellular foams that are biodegradable so that they could then be used in tissue engineering and guided tissue regeneration. Microcellular foams are produced through three ways: the batch mode of microcellular process, the supercritical mode, and the continuous mode. Factors that affect the final foam density in these processes include saturation pressure, foaming temperature, and desorption time. The dependence of foam density on saturation pressure and foaming temperature has been well documented in microcellular foam literature. However, recent results in the continuous mode microcellular process have suggested that desorption time can also play an important role in controlling foam density [5]. This study explores the effect of desorption time on the foam density in the batch process for the following polymers: ABS, CPET, and PC.

2. METHODS

2.1. Materials

A sheet of acrylonitrile-butadiene-styrene, or ABS, manufactured by GE Plastics (CYCOLAC: GPX3700) with a thickness of 0.065², was cut into 1² x 1² square specimens. A sheet of crystalline polyethylene terephthalate, or CPET, manufactured by Eastman Chemicals with thickness of 0.033², was cut into similar size square pieces as ABS. Polycarbonate (Lexan 9030), or PC, came in circular discs with diameter of 1.25² and thickness 0.059².

2.2. Sorption Procedure

A schematic for the batch mode of microcellular process is illustrated in Figure 1.

Pieces of plastic materials were put into the pressure vessel and pressurized with carbon dioxide gas for a certain amount of time until they were saturated. This time, also known as the saturation time, is defined as the time re-
quired by the plastic polymer to absorb the maximum amount of gas in its structure. Carbon dioxide was chosen as the saturation gas due to its high solubility in polymers. For the current study, three randomly chosen representative pieces of each type of polymers were closely monitored during saturation in order to measure the amount of CO$_2$ absorbed by the samples. The weight of each control specimen was measured before saturation in the pressure vessel (virgin weight), and measured again at least once a day as the experiment progressed. The amount of CO$_2$ absorbed by the polymers was calculated by subtracting the virgin weight from the weights of the polymers at different stages of the experiment. The amount of CO$_2$ absorbed in proportion to the virgin weight was graphed with respect to time in the pressure vessel. This graph would indicate the saturation level of the polymers (Figure 2). Once the specimen was saturated, it was taken out from the vessel to be foamed. Another useful information that could be extracted from the saturation graph is the average diffusion coefficients of each polymer. They could be calculated by the following equation [6]:

$$D = \frac{\pi R^2}{16}$$  \hspace{1cm} (1)

where D = diffusion coefficient (cm$^2$/sec); R = slope (cm$^2$/sec); and t = elapsed time (sec). The saturation data not only acted as an indicator that showed whether the polymers were saturated, but also characterized the property of each polymer.

### 2.3. Foaming and Density Measurement

After the polymers were saturated, they were taken out from the pressure vessel and allowed to lose gas under normal atmospheric pressure for different lengths of time, which is known as desorption time. When the polymers moved from a high pressure environment inside the pressure vessel to a lower pressure environment of atmosphere, the gas originally trapped inside the polymers escaped as a function of time. At different desorption times, one or more pieces of each polymer were put into a hot water or glycerin bath for 5 min where the chosen temperatures were maintained by temperature controller. As a result of thermodynamic instability created by the high temperature, the plastic materials foamed. At a temperature above the polymer’s glass transition temperature, the structure of the polymer softened. The gas diffusing out of the polymer forms small bubbles inside the polymeric structure. These bubbles have sizes ranging from 1 mm to 10 mm. Due to their small sizes, they are called microcellular bubbles. After drying for several days, the foamed polymer density was measured by a precision balance based on Archimedes principle. The ASTM Standard D792 was followed during the density measurements. The relationship of final foam density and desorption time was plotted.

In this experiment, the three different polymers were saturated at the same pressure but foamed at different temperatures. Due to equipment limitation, the highest pressure available was 5MPa. Since higher pressure forces more gas into the polymers, 5MPa would allow maximum number of foaming experiments to be conducted at varying desorption times. The foaming temperatures were chosen from previous experimental data such that the final foam density would be approximately 60% of the virgin density, its density before going through the batch process. For ABS, previous data showed that at 5MPa, two foaming temperatures, 63°C and 115°C, both gave the same final relative density [7]. For CPET, experiment demonstrated that foaming temperature of 80°C gave the desired foam relative density [8]; and for PC, 90°C was most appropriate [9].

**Figure 2**

Sorption plot of each polymer pressurized at 5MPa. Notice that the polymers reach a saturation point when no more gas could be absorbed, independent of how much longer they are in the pressurized environment.
3. RESULTS AND DISCUSSION

3.1 Sorption Results

Figure 2 shows the sorption of carbon dioxide by the three different polymers (ABS, CPET, and PC) as a function of time. Since polymer thickness is an important factor that affects diffusion rate, the results are normalized with respect to thickness, as shown in Figure 3.

Figure 2 shows that the three polymers possess the same overall shape. A steep increase of gas absorption is shown in the beginning and a gradual leveling off starts after the fifth or sixth data point. ABS shows an abrupt saturation point, whereas CPET and PC have more gradual saturation.

Figure 3 shows the normalized sorption plot where thickness of each polymer is taken into account. The slope of the linear section is proportional to the diffusion constant of the polymer.

In Figure 3, the last data point of CPET has its x-axis twice as large as that of the last data point of ABS or PC. This is because CPET is approximately half as thick as ABS or PC. Since thickness is the parameter on the denominator of the x-axis unit, the thinner the polymer the wider spread would the data be. Also note that the normalized plot generates a linear slope with the first five or six data points, which is proportional to the diffusion constant of the corresponding polymer. The steeper the slope, the faster the gas can diffuse through the polymer. The advantage of normalizing the sorption plot with respect to thickness is that we can compare the diffusion constant of each polymer more efficiently. Table 1 is a summary of the diffusion constants obtained from Figure 3 and a comparison to the literature values.

The slope $R$ is calculated from the first five data points of Figure 3; the diffusion constant $D$ is computed by using equation 1. Compare with the literature values, the diffusion constants obtained from this experiment for both ABS and PC are 100 times slower. It is unclear as to what cause the discrepancies between the diffusion constants obtained in this experiment versus those that were presented in the literature. However, one possible explanation could include the production of the materials by different companies.

3.2 Foaming and Density Results

The foaming results are shown in terms of relative density, which is the density ratio of the foamed material to the virgin material. The relative density of the final foam versus desorption time of ABS at two different temperatures, 115°C and 63°C, is shown in Figure 3.

Murray et al. illustrated in his experiment that at 5MPa, both 63°C and 115°C gave the relative density of 63%. The result from this experiment was slightly lower at approximately 50% and 55%. ABS samples at 63°C and at 115°C contain the same amount of gas; however, at higher foaming temperature, more thermo-energy allows the gas inside the structure to foam the polymer. Thus, at a longer desorption time, we would expect ABS samples to foam at 115°C but not at 63°C. The final relative densities of the 63°C group increased from 50% to 99% within the first 20 h of desorption. The 63°C group stopped foaming after 20 h, while the 115°C group still show foaming up to 144 h of desorption.

At the same foaming temperature, a polymer that contains more gas results in more bubbles in the final structure and hence a lower density. Thus, as desorption time increases, more gas diffuses out from the polymeric structure, resulting in higher density of the final foam. Normally, a logarithm-like trend is expected, such as what is shown

![Normalized Sorption Plot](image)

**Figure 3**

Normalized sorption plot. Each polymer was pressurized at 5MPa. Polymer thicknesses are 0.065 inch for ABS, 0.033 inch for CPET, and 0.059 inch for PC.

| Table I |
|-------------------|-----------------|-----------------|
| **Polymer** | **R (cm^2/sec)** | **D (cm^2/sec)** | **Literature value** (cm^2/sec) |
| ABS | 1x-4 | 1.95x-9 | 1.35x-7 |
| CPET | 2x-5 | 7.65x-11 | NA |
| PC | 4x-5 | 4.11x-10 | 4.75x-11 |

However, those foamed at 115°C show an interesting pattern: a “dip” in relative density during the first 3 to 4 days of desorption. The final foam density started with approximately 55% of the virgin density. It decreases to nearly 20% of the virgin density before increasing slowly again as more gas escapes from the polymer.

When a saturated polymer is foamed at a temperature much higher than its $T_g$, thermo-expansion occurs too fast and bubbles collapse. After a few hours of desorption, less gas is contained inside the polymer during foaming, and more un-ruptured bubbles are locked into the foam structure. Thus, comparing with the 115°C group sample foamed at 0 desorption time, those foamed a few hours later have a lower density due to more un-ruptured bubbles in the structure.

Both CPET and PC present a result similar to the 63°C ABS group. The relative density of the final foam versus desorption time of CPET at 80°C is shown in Figure 4.

The final foam density increased from approximately 60% to 90% within the first day, and slowly increased to 99% by the fourth day. This gradual rise in relative density provides researchers with more control of obtaining a desired final density by using desorption time as a factor.

The relative density of the final foam versus desorption time of PC at 90°C is shown in Figure 5.

The graph shows that the relative densities of PC range from 67% to 99% within the first 20 h of desorption. Although PC has similar density ranges as the other polymers, it does not increase in a gradual pattern such as that of CPET. This change, taking place in relatively short desorption time, might suggest a harder control of the desired final foam density using desorption time as a factor.

4. CONCLUSION

Through the batch mode of microcellular process, ABS, CPET, and PC were pressurized at 5MPa and foamed in heated water or glycerin bath at different temperatures under atmospheric pressure. The results showed that desorption time has an effect on final foam density. All three polymers had approximately the same range of final relative densities, 60% ~ 90%. However, CPET, foamed at 80°C, changed its densities during the widest range of desorption time than both ABS at 63°C and PC at 90°C. ABS
foamed at 115°C and showed an interesting pattern; the relative density decreased from 55% at 0 desorption time to about 20% at 28 h of desorption. These results have supported the idea of controlling microcellular foam density by varying desorption time.

5. RECOMMENDATION

For future work, the effect of desorption time on foam density can be characterized for biodegradable polymers, such as PLGA (Poly (lactic-co-glycolic) acid). Materials with larger thickness, saturated at various pressures and foaming temperatures should be explored to completely understand the effect of desorption time on the density and microstructure of different microcellular polymeric foams.

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REFERENCES

Over Expressing Integrin Beta_3 Increases Cellular Adhesion in Smooth Muscle Cells

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Abstract: Small diameter vascular grafts are prone to thrombosis and occlusion, which may be prevented by lining vascular grafts with endothelial cells. It would be desirable to increase the adhesion in endothelial cells to line vascular grafts and prevent thrombosis. Integrin over-expression may increase cellular adhesion. To test this hypothesis, we retrovirally infected smooth muscle and endothelial cells to stably over-express the Beta_3 integrin (B3). The effects of over-expressing B3 were studied using adhesion assays, phase contrast microscopy techniques, and cell number studies. Results showed that in smooth muscle cells, over-expressing the B3 integrin increased cellular adhesion to osteopontin compared to vector control cells. B3 over-expression also increased cellular adhesion to fibronectin at low concentrations. The smooth muscle cells were plated on osteopontin, more spreading was seen in cells that over-expressed B3 compared to the vector control. The smooth muscle cells were healthy as over-expressing B3 did not affect cell number or strongly affect other integrins. Therefore, over-expressing an integrin can increase adhesion.

1. INTRODUCTION
It is necessary for blood to flow smoothly through arteries and veins so that body tissues can be properly nourished. Unfortunately, because of injury, diabetes, or atherosclerosis, blood vessels can become damaged or occluded. Occluded arteries are commonly treated by balloon angioplasty and stenting. In this process, a catheter tipped with a balloon is inserted into the occluded vessel. The plaque is pressed against the wall of the vessel, and the vessel is then held open with a stent [1]. Sometimes the damage is too great for this method, and part of the vessel must be replaced. Currently, damaged arteries can be replaced with native vessels of similar size. If appropriate sized vessels are not available, vascular grafts are used in their place [2]. An optimal vascular graft should be biocompatible, resistant to infection, nonthrombogenic and easy to suture [3]. This ideal graft does not exist, however. Many studies are looking to optimize current vascular grafts.

Replacing blood vessels has been successful depending on the size of the vascular grafts. This process works well for large diameter grafts (greater than 6 mm); however, small diameter vascular grafts are susceptible to occlusion caused by blood clots [4]. It is hypothesized that endothelial cells prevent thrombosis in native vessels [5]. Endothelial cells line blood vessels and secrete factors that inhibit thrombosis. People have tried to line vascular grafts with endothelial cells, but the cells are not retained upon exposure to blood flow. The newly exposed vascular graft is then prone to thrombosis [5]. It would be desirable to increase the likelihood of these cells to remain attached to the biomaterial or in other words increase their adhesion.

Adhesion is the act of cells linking to proteins and other components in the extra cellular matrix (ECM). Adhesion allows cells to stay in their appropriate positions and interact with other cells through cell surface receptors or integrins. Adhesion through integrins has a fundamental importance in cellular systems. Integrins are heterodimeric transmembrane proteins that link

![Figure 1](image-url)

**Figure 1**
FACS Results. (a-c) Green fluorescence for GFP, (d-f) Red fluorescence for B3 surface expression.
This study we have retrovirally infected smooth muscle cells (SMCs) as proof of principle, and rat aortic endothelial cells (RAECs) to overexpress the beta_3 (B3) integrin. Our goals were to determine if B3 over-expression increased cellular adhesion.

The cells were retrovirally infected with the cd3a construct, which encodes the genes for B3 and green fluorescent protein (GFP) or the vector control, which encodes GFP only. Infection rate could be measured based on the percent of GFP-positive cells. Analysis of these cells consisted of flow cytometry, adhesion assays, microscopy, and cell number assays.

2. MATERIALS AND METHODS

2.1 DNA

The PBMN-eGFP retroviral vector was obtained from Gary Nolan (Stanford University). The mammalian expression vector containing the cDNA for human integrin B3 (cd3a) was cut with the restriction enzyme XhoI [9]. The cd3a fragment was purified and ligated into XhoI-cut PBMN-GFP. Correct orientation was verified by restriction digest and DNA sequencing.

Mammalian expression plasmids encoding human B3 truncated at amino acids 752 and 755 have been previously described [10]. The mutated B3 DNA was amplified by polymerase chain reaction and inserted into the Topo TA cloning vector (Invitrogen). The B3 segment was removed by restriction digest with BglII and XhoI, purified, and ligated into the BglII/XhoI cut PBMN-GFP retroviral vector. Clones were verified by restriction digest and DNA sequencing.

2.2 Cell culture

The Phoenix retroviral packaging cell line was obtained from Gary Nolan (Stanford University).

- WKY- eGFP –rat smooth muscle cells that express GFP vector control cells.
- WKY-cd3a –rat smooth muscle cells that over-express the B3 subunit also express GFP.
- WKY-752 –rat smooth muscle cells that over-express a mutated B3 subunit truncated at the 752 amino acid sequence.
2.3 Retroviral Infection

For transient transfection of the packaging cell line, 5x10^6 Phoenix cells were plated in 100 mm dishes. Twenty-four hours later they were transiently transfected with 24 µg of plasmid DNA and chloroquine (25 µM) to prevent lysosomal degradation. The packaging cells were moved to 32°C because the virus was heat sensitive. The virus was harvested by collecting the media from the packaging cells 24-48 hours after transfection. Target cells were infected with the virus by adding media containing retrovirus to the target cells and spinning at 1100g for one hr at 32°C. To maximize infection, cells were infected every 24 hours for a total of three infections.

2.4 Flow cytometry

As described in Smith [11], one million cells were incubated in primary antibody SZ21 (mouse anti-human B3) or mouse IgG diluted 1:50 at 4°C for 30 min. The buffer that allowed the antibody to bind was PBS/0.2%BSA/0.02%NaN3. The buffer was then used to wash the cells. Then the secondary antibody was allowed to bind also at 4°C for 30 min. The secondary antibody [PE Anti-mouse IgG (Biomeda) (1:50 dilution)] contained phycoerythrin, a red fluorescent marker. To rid of any unbound antibodies, the cells were washed twice with 1 mL of PBS/NaN3. The cells were fixed with 300 mL of 1% PFA and stored covered at 4°C.

2.5 Adhesive Proteins

Purified bovine plasma fibronectin was obtained from Gibco BRL, and rat tail collagen type I was obtained from Collaborative Biomedical Products. Full-length recombinant rat and human osteopontin were generated as histidine-tagged fusion proteins as previously described [11].

2.6 Adhesion assay

Adhesion assays were performed as described in Liaw [8]. Proteins were diluted in phosphate buffered saline (PBS) to desired concentrations, and protein solution was added to a 96-well ELISA/RIA plate. Proteins were allowed to incubate at 4°C overnight. The proteins were removed, then washed with PBS. PBS was extracted and 10µg/mL of bovine serum albumin (BSA) in PBS was added to block nonspecific binding sites. Cells were prepared by washing with versene then versene-trypsin. The cells were spun down then washed twice with 1 mg/mL of BSA in media without serum. The cells were then resuspended in 1mg/mL of BSA in media without serum. BSA in PBS was removed from wells, and then 30,000-60,000 cells were added to each well. Plates were incubated for two hours at
37°C, and then unbound cells were washed away with PBS. Cells were fixed to plate with 4% paraformaldehyde. Bound cells were stained with 0.5% toluidine blue in 4% paraformaldehyde. Plate was washed with ddH₂O until no blue dye was seen in water. Stain was solubilized with 1% Sodium Dodecyl Sulfate. Assay was quantified with an ELISA plate reader at 630 nm.

2.7 Microscopy experiment
In order to simulate conditions of the adhesion assay, osteopontin, at the desired concentrations, was added to a 6 well plate and incubated overnight at 4°C. The wells were blocked with 10mg/mL of BSA in PBS for one hour at 37°C. SMC’s were resuspended in 1 mg/mL of BSA in media (without serum). To each well, 150,000 cells were added. After a 2-hour incubation, the cells were examined using phase contrast microscopy on an inverted microscope. Microscope specifications: Nikon TE200 Inverted Microscope, Photometrics Cool Snap camera, and 10x objective.

2.7.1 Cell number
To each well of a 6-well plate, 50,000 cells were added. At all time points, cells were trypsinized and counted using a ZIS Coulter Counter. Media was replaced at day 2.

3. RESULTS

3.1 Cells Over Express B3 I
The rat SMC line WKY was retrovirally infected with the human integrin B3 or vector control. Our vector control cells (eGFP) were infected with the virus that only coded for the green fluorescent protein, while the cells that over express B3 (cd3a) coded for B3 over expression as well as GFP. Flow cytometry was used to determine infection efficiency (% GFP-positive) and level of B3 surface expression. Typical results are shown in Figure 1. Both vector control cells and cells infected with the cd3a construct were efficiently infected (97% and 82% respectively). Cells infected with cd3a (92%) over-expressed the B3 integrin on their surface compared to vector control (10%). Cells infected with the mutant B3 constructs also showed over-expression of B3 on their surface (Table I).

3.2 Cd3a Cells Adhere More to Osteopontin than Vector Control
To examine the effects of over expressing B3, adhesion assays were used to quantify amounts of cellular adhesion. Results showed that adhesion to osteopontin (OPN), which binds predominantly to the integrin alpha_v beta_3, is in-
increased in cells that over express B3 (Fig. 2). The proteins used were rat OPN and human OPN. The results showed that there were no significant differences between these two OPN types, but the differences between B3 over-expressing cells and vector control cells were obvious. Since OPN binds primarily to the integrin that was over expressed, more cells were able to adhere because of the increased B3 on the cell surface. Over expressing B3 does increase adhesion to OPN.

3.3 Over-expressing B3 Has No Effect on Cell Number

One question that arose was whether or not over expressing B3 causes effects on the normal characteristics of the cells. To answer this, the cells were examined with a cell number assay. This was to determine if over expressing B3 affected cell growth. The assay counted the number of adherent cells over the course of four days. The results of the cell number assay were analyzed using student t-tests, which found that the differences between the two cell types were not statistically significant, as seen in Figure 8 (p>0.05). The cd3a cells grew at rates similar to the vector control cells; over expressing B3 does not affect cell number.

3.4 Over-expressing B3 Has Little Affect on Other Integrins

After finding that cd3a cells adhere more to OPN, a B3 ligand, questions arose as to whether or not the over expression affects other integrins on the cellular surface. The over-expression of B3, causing alpha_v beta_3 to form, could reduce alpha_v beta_1 levels, and also it could inhibit integrin mediated adhesion. To answer this question, cellular adhesion to collagen I was tested. Collagen I does not bind to B3 integrins, but it does bind to beta_1 (B1) integrins. Results showed that although the differences were statistically significant (p<0.05), the changes were very small compared to the observed differences for OPN (Fig. 3). The cd3a cells showed a little less adhesion to collagen I at the concentrations tested. One possible explanation is that over-expressing B3 can indirectly reduce the amount of alpha_v beta_1 on the cell surface. However, overall expression of B3 has little effect on adhesion to collagen I, which suggests that over-expressing B3 has little effect on other integrins.

3.5 Cd3a Cells Adhere More to FN at Relatively Low Concentrations

FN is a protein that binds to both B1 and B3 integrins [6]. Results from the adhesion assays showed that at low concentrations of FN, cd3a cells had increased adhesion compared to vector control cells; however at higher concentrations, the differences were not statistically significant (Fig. 4). The cells that over-express B3 have more adhesion to FN at relatively low concentrations.

3.6 Over-expressing B3 Increases Cell Spreading

Microscopy experiments were used to determine if over-expressing B3 affects cell shape. The cells were examined on OPN at various concentrations. From the images taken using phase contrast microscopy, the cd3a cells were more spread than the vector control cells (Fig. 5). OPN was plated at 0.25, 2.5, and 25 µg/mL, and significant spreading did not appear in vector control cells until 25 µg/mL of OPN. On the contrary, cell spreading was easily seen in cd3a cells even at the lowest concentrations of OPN. Over-expressing B3 increases cell spreading on OPN. These results correspond with the results found in adhesion assays, where at all OPN concentrations, cd3a cells had increased adhesion compared to vector control cells.

3.7 Truncations of the B3 Tail Decrease Adhesion to OPN

Two other cell types were also used in these studies of integrins on cellular surfaces. These cells had endogenous amounts of rat wild type B3 and also extra B3 that were mutants. The b3 subunit consists of a 762 amino acid sequence [12]. However, the mutants did not have portions of the cytoplasmic tail. The virus that infected these cells had stop codons that ended the translation of the B3 integrin at the amino acids 752 and 755. The DNA mutations were verified by sequencing.

An adhesion assay was performed to determine the effect of the mutation on adhesion in SMC’s. The negative controls were the vector control cells while cd3a cells were
the positive control. The results showed that adhesion in mutant cells fell between the positive and negative controls (Fig. 6). WKY-752 cells were less adherent to OPN than the WKY-755 cells. This means that the distal portion of B3 is required for complete adhesion. Removal of the distal end of the B3 tail reduces adhesion to OPN compared to cells over-expressing wild-type B3.

The effect of these mutations on cell shape was also analyzed. WKY-752 and WKY-755 had reduced spreading compared to cells that over-express wild-type B3. The results from the microscopy also corresponded with the results from the adhesion assay (Fig. 7). Mutations in the B3 tail decrease adhesion and cell spreading on OPN.

3.8 Over-expressing B3 in Endothelial Cells Does not Increase Adhesion to OPN and FN

Our RAEC’s were retrovirally infected to stably over express B3 on their surface. This was verified with flow cytometry (Table I). An adhesion assay was performed and showed that there was no significant difference between cd3a and vector control endothelial cells when placed on OPN and FN (Fig. 9). This is likely because in vitro RAEC’s have a lot of endogenous B3, and the system may be saturated. Therefore, adding more B3 to the surface would have no effect.

4. DISCUSSION

SMC’s that over-express B3 have increased adhesion to OPN than vector control cells. They also adhere more to FN at relatively low concentrations but not at high concentrations. Since cells can bind to FN through both B3 and B1 integrins, we believe that at high concentrations, adhesion through B1-integrins may be masking any effect of B3 over-expression. The adhesion assay on the B1-ligand collagen suggests that over-expression of B3 has a negligible effect on the function of B1 integrins.

This study also shows that B3 over-expression does not have much effect on normal cell characteristics. The cell number study showed no statistical differences between B3 over-expression and vector control cells. When grown on tissue-culture polystyrene in growth media, no difference in cell shape was observed between the two cell types. This emphasizes the fact that the cells over-expressing B3 are relatively healthy. The microscopy study backs up the adhesion assays in that more spreading occurred in cells over-expressing B3. Truncation of the cytoplasmic tail of B3 at amino acids 755 and 752 impairs but does not completely inhibit adhesion and spreading. These results agree with the previous findings that these regions of B3 were necessary for cell spreading in CHO cells [10].

Our studies prove the principle that over-expressing an integrin will increase adhesion. SMC’s were an optimal cell type for the study because they do not have much B3 on their surfaces; however, endothelial cells must be used to line vascular grafts. A preliminary experiment suggests that endothelial cells over-expressing B3 do not have enhanced adhesion to OPN or FN. It is possible that they may show improved adhesion at other protein concentrations or on other B3-ligands. Activation of B3 may also be required to see differences in endothelial cell adhesion. Also, endothelial cells do not express much B3 on their surfaces in vivo [13]. Therefore, it is possible that cells over-expressing B3 would cause more adhesion to OPN or FN in vivo. Finally, over-expressing a different integrin could possibly increase adhesion as well. We have proven the principle that integrin over expression can increase cellular adhesion without adversely affecting the cells.

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Abstract: The FDA currently classifies hydrophilic contact lenses based on the water content and ionicity of the lens polymer content. While this method of classification is useful chemically, it is of little use to the clinician because it does not relate to the lens’ interaction with the eye. A more useful classification system would involve relative lens-eye interaction and be a good predictor of lens’ protein affinity. In this study, we have attempted to correlate lens interaction with an important tear protein, lysozyme, to lens interaction with a cationic dye, methylene blue. The purpose of this study is to investigate the hypothesis that methylene blue is representative of lysozyme in binding mechanism and adherence to hydrophilic lenses. Triplicates of 27 contact lens brands were soaked in methylene blue and observed visually using a rating system (A-C) to assess lens darkness. The lenses were then soaked overnight in water to determine the amount of methylene blue that leaches out of the lens. The absorbance of the water solution was then read in a spectrophotometer. This experiment was then conducted using a lysozyme solution. The lenses were soaked in a lysozyme solution for 30 min., then removed and placed in phosphate-buffered saline (PBS) overnight. The protein concentrations of the lysozyme solution and the wash solution were then determined using spectrophotometry as well as by microBCA assay. Cluster analysis of the methylene blue and lysozyme studies showed no correlation between methylene blue and lysozyme relative to lens interaction.

Future work might include a quantitative method of assessing initial methylene blue penetration as well as utilizing a more reliable method for quantifying lysozyme concentrations, such as radiolabeling.

1. INTRODUCTION

More than fifteen years ago, the FDA developed a hydrophilic polymer contact lens classification table to be used mainly as a guideline for solution interactions safety studies. This table was based exclusively upon the empirical formula presented by the contact lens manufacturer as it pertained specifically to the methacrylic acid monomer and water content when the lens is fully hydrated. The FDA assigned each lens to one of four groups: Group I (low water, non-ionic), Group II (high water, non-ionic), Group III (low water, ionic), Group IV (high water, ionic) [1, 2]. The assumption in using this classification system was that the ionic nature and solution interaction with the lens was implied by one monomer and water content related to the polymer’s properties [3]. Though this classification has been known to be a valuable tool for solution attachment studies, there are certain inconsistencies within a group due to the limited variables and polymer properties considered. A more appropriate classification system to be used in clinical applications would be to assess each lens’ interactive characteristics with respect to tear components, specifically protein.

Inappropriate deposition onto contact lens spoilage causes nearly 80% of all clinically diagnosed contact lens-related malaise [2, 4]. Interaction between the lens and the eye begins almost immediately [5]. Within 1 min, components of the tear fluid begin to interact with the lens and proteins may continue to adhere for up to one week in continuous wear lenses [6]. The initial interaction of tear fluid, composed of mucins, proteins, salts, water, gases, sugars, urea, etc, with the lens is essential in creating and maintaining biocompatibility between the lens and eye [2, 7, 8]. Mucins and proteins initiate adherence to the lens; a primary deposition zone begins to form as proteins deposit, undergo conformational changes, and become irreversibly bound to the lens polymer matrix [2, 8]. Throughout wear-time, further protein deposition occurs resulting in a “coating” over the initial primary zone [2]. When the entire lens is covered in this coating, the pellicle is considered to be complete. This pellicle provides a cushioning effect when a patient-specific thickness is achieved. Problems arise, however, when further, abnormal depositions of mucoproteins, lipids, amino acids, organic debris and microorganisms form over the initial pellicle layer [7, 8]. Abnormal depositions can result in mechanical irritation, disrupted visual perception, corneal staining, conjunctival hyperemia, hazy vision, decreased blinking, increased chance of microbial contamination [2, 5, 9]. Increased deposition can be attributed to patient-specific properties: sub-optimal lens fit, low water component in tear secretions, increased chance of tear fluids, dry eye syndrome, certain medications, decreased blinking, high fat diets, alcohol consumption, and low bodily potassium level [10, 11].

Although human tears contain approximately 60 different proteins, scientists have found lysozyme to be the primary protein that interacts with contact lenses in vivo [1, 7]. The prevalent nature of lysozyme can be attributed to the fact that it constitutes one-third of the total protein com-
position of tears [12]. In addition, lysozyme, a positively charged protein, is strongly attracted via electrostatic forces to the negatively charged methacrylic acid in the hydrophilic lenses [5]. A strong association between lysozyme affinity by the hydrophilic lens polymer and FDA lens classification also exists (e.g., the amount of protein absorbed onto the lenses is directly related to the ionic properties of the lens) [8, 12]. Despite this correlation, oversimplification of this situation should be avoided as there are tear proteins other than lysozyme, such as mucins and lipids, which also interact with the lens in the ocular environment. The literature, however, predominantly points to lysozyme as the major tear moiety of importance in vivo. The logic behind this study was to find a cationic dye that would ionically bind to lenses in an attraction mechanism similar to that of lysozyme. This dye should then be representative of the in vitro interaction of the lens with lysozyme binding site, leading to more simple and accurate assays to determine lens ionicity. We propose that the affinity of a cationic dye for a lens will more accurately describe its ionic nature, for it is based upon the logic of using the polymer as it actually exists (including hydration, crosslinking agents, and other monomers) rather than relying on chemical formula alone. We have investigated the determination of contact lens type using methylene blue, a cationic dye. Additionally, we have compared and contrasted the in vitro affinity of lysozyme and methylene blue for all of the lenses tested. Based on the initial interaction of the polymer with lysozyme, a representative protein for the overall tear components, we foresee a more clinically relevant grouping system based on an in vitro analysis of lens-protein interaction. Because this study assesses the in vitro lens-eye interaction, certain properties of in vivo interaction such as cyclic rewetting, lid force, and blinking habits, are not taken into account [9]. Therefore, our proposed classification is based upon the in vitro representation of the initial lens-eye interaction and should be taken as a starting point in clinical practice where further patient characteristics, such as tear film properties, wear schedule, disinfection habits, atmosphere, medication, and diet should be taken into account when fitting lenses.

2. MATERIALS AND METHODS

2.1 Investigation of lens interaction with methylene blue

2.1.1 Sample Preparation

Triplicates of twenty-seven lenses of varying brands were chosen based on their representation of the major chemical composition of the different groups of the current FDA system. All samples were of equal optical strength and triplicates from the same lot number to maintain unanimity throughout testing. Samples were individually soaked in 12-well microplates (Falcon) for 30 min in 5 ml of 0.2% methylene blue dissolved in water. The lenses were then removed and placed in 6-well microplates (Falcon) containing 10 ml of double distilled water in each well. The degree of methylene blue penetration (darkness of the blue color) was observed visually and recorded according to the following scale: “A” to describe a dark blue lens appearance, “B” for an intermediate blue lens, and “C” for a light blue lens. Microplates were placed on a shaker overnight, after which the degree of methylene blue penetration was once again observed visually and recorded. The following scale was employed: “1” refers to a dark blue lens with no leaching of dye into the water, “2” refers to a dark lens with slight leaching of dye, and “3” describes a lighter lens with a high degree of leaching into the surrounding water.

2.1.2 Spectrophotometry

A spectrophotometer (Cecil CE2041) was used to assess the amount of methylene blue released into the water wash. A 1 ml aliquot from each of the lens washings was pipetted into a disposable cuvet (VWR) and the absorbance was read at 668 nm and recorded.

2.2 Investigation of lens interaction with lysozyme

2.2.1 Sample Preparation

All lenses used in this study were FDA approved and classified using the grouping system based on ionicity and water content. All samples were new and unopened from original packaging. Three samples of 27 different contact lens brands were removed from packaging and immediately placed individually in 12-well microplates (Falcon) for 30 min in 5 ml of 20 mg/ml lysozyme (Sigma-Aldrich) dissolved in phosphate-buffered saline (PBS). Samples were then dip-rinsed in PBS to remove loosely adhering protein. The lenses were then immediately transferred to 6-well plates containing 10 ml of PBS in each well. Plates were shaken overnight.

2.3 Spectrophotometry

Solutions of known concentrations of 0, 10, 20, 30, 40, 50 µg/ml lysozyme were analyzed over a period of days before experimentation to calibrate the data in a standard curve of absorbance vs. concentration. 3 ml aliquots of the each of the lens washings were removed from the 6-well plates and placed in a cuvet. Aliquots from the initial lysozyme soaking solution were diluted 1:1000 in order to accommodate the sensitivity range of the equipment [9]. The absorbance of each sample was measured at 280 nm in a spectrophotometer (Cecil- CE2041) to determine the concentration of lysozyme in the washings. This was calculated by relating the wash absorbance to the standard curve.
2.4 Micro-BCA Assay

A micro-bicinchoninic acid protein assay (micro-BCA assay) was used concurrently with the spectrophotometer analysis to validate protein concentration of the washings. Because all samples were prepared in PBS, the microBCA assay was chosen as a reliable protein quantification method for its simplicity, sensitivity, and tolerance to buffer solutions within the samples [12]. The BCA method utilizes the biuret reaction in which a peptide bond in the protein reduces Cu²⁺ to Cu⁺ in the solution environment [12]. This reduction is achieved through the addition of CuSO₄ to the reagent mixture. While the biuret reaction involves a color change that can be observed visually, the absorbance can be quantitatively analyzed using a spectrophotometer. The microBCA assay used in this experiment also takes advantage of the color change involved in the biuret reaction, but involves a more concentrated reagent so as to be more effective on dilute protein solutions. The micro-BCA assay was meant to be used at concentrations in the 0.5-10 µg/ml region. Because the post 30 min soak solution is presumably at a concentration close to 20 mg/ml, all post 30 min solutions were diluted 1:1000 to fit into the sensitivity range of the microBCA assay. The protein concentrations were calculated using a bovine-serum albumin standard curve, a common and reliable BCA assay standard. The micro-BCA protocol involves exposing a set volume of sample to an equal volume of reagent. When exposed to a particular temperature, the chemical reagent initiates the biuret reaction and the color change follows [13]. In this experiment, 100 µl aliquots from each of the lens washings were pipetted into a 96-well microplate (Falcon 3915) along with a set of BSA standards for calibration of the data. The micro-BCA assay reagents were combined and pipetted at 100 µl/well. The plate was placed on a shaker for 1 min and then incubated at 60 ºC for 1 h. The plate was cooled for 15 min at RT and absorbance was measured using a microplate reader (Molecular Devices VersaMax) at 560 nm.

3. RESULTS

3.1 Investigation of lens interaction with methylene blue

3.1.1 Cluster Analysis

Hierarchical cluster analysis was used to quantitatively categorize lenses according to observations of lens and solution appearance after the initial wash, the next day wash, and the absorbance of the next day wash solution. The cluster analysis groups lenses into clusters, so that lenses in each cluster are more similar to each other than they are to lenses in other clusters. The clustering procedure was based on a proximity matrix of squared Euclidean distances between pairs of lenses. Linkage was determined by between groups linkage.

The results of the cluster analysis are depicted as a dendrogram (Fig. 1). Cluster distance is indicative of the degree of similarity between clusters of lenses as determined by similarities in lens and solution appearance following dye exposure and subsequent washes. Clusters linked by the smallest distance are the most similar to one another, as distance is inversely proportional to similarity. Distinct groups are indicated by a sharp increase in the distance at which clusters are joined in the dendrogram. Further aid in quantifying the number of clusters determined by the analysis is provided by scree analysis. The distinct clusters are identified as a “bend” toward the horizontal beyond three clus-

![Figure 1](image-url)
The degree of integrity of each cluster with regard to grouping of replicates of lenses is excellent; all replicates for any given lens are located in the same cluster.

There were no differences in water content between clusters (Fig. 2, Kruskal-Wallis one-way analysis of variance, \( H=1.2, \text{df}=2, P=0.527 \)).

FDA groupings are imperfect predictors of classification of a lens in a given cluster. FDA Groups 3 and 4 are exclusive to cluster 1. FDA Group 2 lenses were categorized in clusters 2 and 3. FDA Group 1 lenses were dispersed relatively evenly in all cluster. See Table I for a representation of FDA groups versus cluster analysis.

### 3.1.2 Characteristics of the clusters

Kruskal-Wallis one-way analyses of variance were used to compare the clusters for each observation of dye adherence. Observations were averaged across replicates for each lens. This non-parametric method was used because observations were not distributed normally.

Based on the cluster analysis, it is clear that clusters represent three distinct patterns in dye adherence to the lens with cluster 1 containing the most adherent lenses (= ‘attractive’) and cluster 3 the least adherent lenses (= ‘repellent’). Lenses in cluster 2 fall in between these two extremes (= ‘neutral’). These differences are apparent after the initial wash (\( H=21.5, \text{df}=2, P<.0001 \), Dunn’s method of post hoc pairwise comparisons, \( P<.05 \)) in which lenses in clusters 1 and 2 are significantly darker and wash solutions lighter compared to lenses in cluster 3. In contrast, results for cluster 2 lenses either fell between those for cluster 1 and 3 (Lens and Solution Condition: Next Day Wash, \( H=20.4, \text{df}=2, P<.001 \), Dunn’s Method of post hoc pairwise comparisons, \( P<.05 \)) or are not significantly different from results for cluster 3 (Lens Condition: Next Day Wash, \( H=25.5, \text{df}=2, P<.001 \); Solution Absorbance: Next Day Wash, \( H=19.9, \text{df}=2, P<.001 \), Dunn’s Method of post hoc pairwise comparisons, \( P<.05 \)).

### 3.1.3 Investigation of lens interaction with lysozyme

For the lysozyme investigation, it appears that the lenses statistically cluster themselves into 3 different groups, denoted A,B,C in Table II. However, these new groups are entirely unrelated to the clusters from the methylene blue investigation. One cluster consists entirely of one CSI lens (one with a next day absorbance value of .907, quite different from any of the other lenses so its not surprising it is a distinct cluster). The other two CSI lens are not grouped with this one. Another cluster consists entirely of the three Permalens samples. These had the second highest next day absorbance values (~.340), much higher than the remaining lenses. In the methylene blue study, the Permalens samples were in cluster 1 (attractive). Finally, all other lenses clustered together. These lenses have similar low 30 min and next day absorbance readings. There are no sub-clusters within this cluster: all of these samples are very similar.

## 4. DISCUSSION

Based on the statistical analysis of the methylene blue and lysozyme studies, it is apparent that these two clustering methods did not agree. This could be due to two different scenarios. It is possible that the hypothesis is incorrect and that lysozyme does not interact with the lenses in the same manner as methylene blue. Therefore, methylene blue cannot be used to represent lysozyme in further experiments. Another possibility is that the comparison of the two investigations is not appropriate. For example, in the methylene
blue investigation, there is a qualitative component involved in the analysis of the initial methylene blue penetration into the lens. The lysozyme investigation may be flawed with respect to the lack of this qualitative assessment. Alternatively, it might be more desirable to include a quantitative measurement of the initial methylene blue penetration into the lens, possibly using spectroscopy. A statistical analysis would be easier to perform on data that is more cohesive with regards to experimental design. In other words, both of the investigations must be almost exact replicas of each other with the only difference being the methylene blue vs. lysozyme.

Future work might involve including a quantitative measurement of the initial methylene blue penetration and a more accurate assessment of lysozyme absorption. Radiolabeling is a reliable method for protein quantification and could be used in the original experimental design.

The observation that minimal amounts of lysozyme were released from the lens matrix can be attributed to an irreversible penetration of the protein into the lens polymer matrix. In some instances, this irreversible penetration can be further strengthened by self-association between the lysozyme molecules. Self-association is probable in a situation such as this when lysozyme is the only protein in the surrounding solution and the only extraneous protein bound to the lens. After initial lysozyme adsorption, the adsorbed protein molecules shield the lens from further protein adherence. Therefore, further protein deposition is more likely to occur through self-association with already adsorbed lysozyme and not the actual lens material. High water content, ionic lenses (FDA Group IV) tend to acquire protein penetration more rapidly than other lens types because of the increased electrostatic attraction of the charged protein to the ionic lens material, as well as the increased pore size of the lens. Low water content, non-ionic lenses (FDA Group I) are more prone to protein surface adsorption, which has the potential to be irreversible [14]. In theory, the pellicle of non-ionic lenses is a thinner layer that may be less comfortable to the wearer as opposed to the thicker pellicle layer of ionic lenses. However, it is not yet clear whether the thickened pellicle of ionic lenses enhances biocompatibility as much as it enhances inappropriate protein deposition. Certainly, comfort level is directly related to patient-specific factors in which case certain lens characteristics (high water vs. low water, ionic vs. non-ionic) may create adverse reactions contrary to what theory predicts [11].

### 5. CONCLUSION

In the clinical arena, it is very valuable to know when a lens type is causing a patient to encounter an adverse reaction in order to select an alternative lens. The level of inappropriate protein depends on the tear-lens interaction and varies based on polymer content, ionicity, and protein affinity [2]. Because of this variance, an improved classification system may indeed prove clinically desirable to assist in changing from one lens type to another, thereby modifying subsequent tear interactions. It is imperative that the clinician understands the impact of varying polymer charge to modify tear interactions. Clinicians may want to further

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modify lens thickness, edge design, or other variables in order to maintain a biocompatible surface with minimal inappropriate deposition of tear moieties. Of course, lens compatibility is most heavily limited by the patient’s own tear properties and the individual’s interaction with the lens [2]. In conclusion, the classification of contact lenses according to potential in vivo ionic interactions with human tears is integral to the process of achieving and manipulating biocompatibility of a contact lens in the eye [8]. For this reason, a new classification system would be advantageous.

ACKNOWLEDGEMENTS

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REFERENCES

Intracellular Uptake of Methotrexate Surface Modified Superparamagnetic Magnetite Nanoparticles

Adam Oliver, Nathan Kohler, Miqin Zhang
University of Washington Engineered Biomaterials, University of Washington, Seattle WA 98195

Abstract: Superparamagnetic nanoparticles as MRI contrast agents can be fluorescently tagged and directed by an external magnetic field. All these characteristics make them ideal candidates for drug delivery vessels. In this experiment superparamagnetic nanoparticles were successfully synthesized and surface modified with the SAM (3-aminopropyl)-trimethoxysilane, folic acid, methotrexate and fluorescein. The size and dispersion of the particles were verified with AFM, and the surface modification was characterized using FTIR. The nanoparticles were surface modified to quantify cellular uptake of methotrexate surface modified nanoparticles, unmodified nanoparticles, and nanoparticles surface modified with folic acid.

1. INTRODUCTION

The use of superparamagnetic magnetite nanoparticles in cancer therapy has been demonstrated in several different proposed applications. Among these applications are vehicles for tissue specific drug targeting [1], contrast agents in magnetic resonance imaging (MRI) [2], and hyperthermia [3]. Previous work has shown the ability of nanoparticles surface modified with folic acid to specifically target rapidly multiplying cancer cells. The proposed mechanism for this targeting is receptor mediated endocytosis. Folic acid is essential in the replication of DNA because methylating role tetrahydrofolate and the folic acid cycle. Rapidly multiplying cells, such as cancer cells thus require a large quantity of folic acid and as a result folic acid receptors on their surfaces are over expressed relative to normal cells [4]. This over-expression is believed to cause folic acid surface modified nanoparticles to be internalized by cancer cells and other rapidly multiplying cells with a higher frequency than they are internalized in others. The folate receptor is not only a tumor marker, but has also been shown to efficiently internalize molecules coupled to folate [5]. This has possible applications in drug delivery as well as in identifying new budding tumors through MRI while they are still small.

Methotrexate (MTX), commonly used as an anticancer drug to treat acute leukemia, is a structural analog to folic acid. It is an anti-metabolite with an antagonistic effect on folic acid metabolism. Its method of action is not completely certain, but it is believed to function as an anticancer drug by binding to the coenzyme dihydrofolate reductase, inhibiting the binding of tetrahydrofolate and halting the folic acid cycle. As a result, methotrexate has systemic toxicity.

In our experiment we covalently bonded methotrexate to the nanoparticle via a (3-amino)-trimethoxysilane self-assembled monolayer (SAM) on the nanoparticle surface. In this bonded state it is believed the methotrexate is unable to react with the dihydrofolate coenzyme and is thus safe in the body. We also plan to quantify the uptake of methotrexate surface modified nanoparticles (MSN) to test if methotrexate facilitates endocytosis of the nanoparticles via the folate receptor as its analog folic acid does. We also plan to test if once internalized into an endosome in the cancer cell the methotrexate can be successfully released from the nanoparticle as a function of the greater pH in the endosome relative to the rest of the body. Once released in the cell, the methotrexate would bind to dihydrofolate reductase, halt the folic acid cycle, terminate DNA replication and kill the cancer cell. If these tests are successful magnetic nanoparticles could serve to be a very effective means of delivery.

It is our expectation that modifying the surface of nanoparticles with methotrexate will facilitate uptake of nanoparticles into specific cancer cells through receptor mediated endocytosis just as modifying the surface with folic acid was previously shown to do. This will combine specific drug delivery with increased resolution for diagnosis. Fourier transform infrared spectroscopy (FTIR) was used to characterize the immobilization of the MTX and folic acid on the nanoparticle surface. The uptake of MTX surface modified nanoparticles into cancer cells will be performed using breast cancer (BT-20) cells with folic acid surface-modified nanoparticles and unmodified magnetite nanoparticles as controls. Fluorescence and confocal microscopy, and inductively coupled plasma emission spectroscopy (ICP) were used to visualize and quantify the uptake of nanoparticles into the cells.
3.2 Modification of nanoparticles with methotrexate

In order to modify the surface of the nanoparticles with methotrexate, it is necessary to covalently graft an amine-terminated SAM to the nanoparticle surface. One g of nanoparticles in D.I. water was separated from solution under the influence of a large rare earth magnet. The particles were then resuspended into 50 mL of acetonitrile under strong vortexing. The solvent exchange was then repeated an additional three times before the resulting suspension was poured into a round bottom flask. The particles were then separated from the acetonitrile solvent and resuspended dispersed in 3 mM (3-aminopropyl)trimethoxysilane in acetonitrile. The resulting suspension was then sonicated for four hours at 60°C. The SAM modified nanoparticles were separated from solution under the influence of a rare earth magnet and resuspended in dimethylsulfoxide (DMSO). The particles were then washed an additional three times in DMSO, 1.5 mL 15 mM NHS and 1.5 mL 75 mM MEDC solution in water using triethylamine as a catalyst. The pH of the solution was adjusted to 9.0 and the resulting suspension was incubated at 37°C for four hours. The MTX grafted nanoparticles were then separated from solution under the influence of a rare earth magnet and resuspended in a solution of 70%

2. MATERIALS

The superparamagnetic nanoparticles were synthesized using the given procedure. The diameter of the nanoparticles was measured to be about 30 nm. N-hydroxysuccinimide (NHS) was purchased from Shearwater, Huntsville Alabama. Methotrexate, fluorescein, (3-aminopropyl)trimethoxysilane, and folic acid were purchased from Sigma-Aldrich, St. Louis, MO. Breast cancer cells (BT-20) and RPMI-1640 medium were purchased from American Type Culture Collection (ATCC TIB-186, Rockville, MD).

3. METHODS

3.1 Synthesis of Magnetite Nanoparticles

3.09 g FeCl₂ * 4H₂O and 5.2 g FeCl₃ were added to a solution of 0.85 mL HCl and 25 mL deoxygenated D.I. H₂O. The resulting solution was then slowly dripped using a micropump into 500 mL of 1.5 M NaOH under sonication. This caused oxidation and the formation of tiny magnetite particles. The precipitated nanoparticles were separated from solution using a large rare earth magnet. To wash the particles and lower the pH, the basic supernant was removed and 500 mL D.I. water was added. This washing process was repeated approximately 5 times until a pH of 9.0 was achieved. The solution was sonicated and the nanoparticle suspension was then divided into 8x50 mL centrifuge tubes.
ethanol in D.I. water to maintain a sterile environment.

Figure 2 is a diagram illustrating the chemical steps taken to graft the methotrexate to the surface of the nanoparticles.

3.3 Modifying magnetite nanoparticles with folic acid-fluorescein

The magnetite nanoparticles modified with MTX obtained above were used for further attachment of fluorescein (Figure 3). The nanoparticles were added to a mixture of 5mL 0.015M fluorescein solution in methanol, 1.5 mL 15 mM NHS and 1.5 mL 75 mM EDC solution in water, using triethylamine as a catalyst. The pH was adjusted to 9 and incubated at 37°C for 4 h. The particles were then separated from suspension under the influence of a rare earth magnet and re-suspended in 70% ethanol solution to maintain sterility.

3.4 Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were obtained using a Nicolet 5-DXB FTIR spectrometer with a resolution of 8/cm. To characterize the amine SAM and MTX on the surface of the nanoparticles, 2 mg of magnetite nanoparticles were dried overnight under vacuum and mixed with 190 mg of KBr. The mixture was then pressed into a pellet for analysis.

3.5 Transmission electron microscopy (TEM)

To characterize particle size and ensure particle dispersion, 1 mL of the nanoparticle suspension was diluted into 20 mL of pH 9 D.I. water. The resulting suspension was then sonicated for 20 min and 300 mesh silicon monoxide coated grids were then dipped into the solution and dried overnight under vacuum.

3.6 Cell Culture

Human breast cancer cells (BT-20) were used in these experiments. The cells were grown in 75 cm² flasks containing 10 mL of RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mM amino acids, 50 IU/mL penicillin and 50 µg/mL streptomycin. The media was changed every third day. The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

3.7 Inductively coupled plasma emission spectroscopy (ICP)

To later quantify the uptake of the nanoparticles into the BT-20 cells, the iron concentrations of all the different test solutions were measured using ICP. The solutions were simply separated into separate tubes, and adjusted so that the concentrations would be the correct magnitude for measurement. After the cells are put into a solution containing nanoparticles, the nanoparticle uptake will be measured by calculating the iron concentration difference in the solutions before and after cell exposure.

4. RESULTS

The AMF scans in Figure 4 show that the particles are well dispersed and have a diameter of about 30 nm. This dispersion is important if the nanoparticles are to be successfully surface modified. Some moderate clumping can be seen in Figure 4B, the unmodified nanoparticles, but it is not extensive.

Figure 5 contains the FTIR spectra of silane modified and silane-folic acid modified nanoparticles, the unmodified native nanoparticle spectra as well as the folic acid spectra. Figure 6 contains the FTIR spectra of the silane modified and silane-methotrexate modified nanoparticles, the spectra of the native nanoparticles, and the methotrexate spectra. In the C spectra of both Figures 5 and 6 there is a large peak at 1040-1030 cm⁻¹. This is the silicon-oxide peak and is strong evidence that the SAM (3-aminopropyl)-trimethoxysilane was effectively bonded to the nanoparticle surface. In the A spectra of Figures 5 and 6 there was a large increase in the size of the relative peaks around 1650-1620 cm⁻¹, corresponding to the amide I band. This is evidence that the peptide bond is forming between the SAM-amine and the folic acid, as well as between the SAM-amine and methotrexate. This bonding indicates that grafting folic acid and methotrexate to the surface of the nanoparticle was successful.
5. DISCUSSION

Superparamagnetic magnetite nanoparticles were successfully synthesized. Their size and dispersion are acceptable for cellular uptake and surface modification, this was verified visually though AFM. Surface modification of the nanoparticles with methotrexate, folic acid and fluorescein was also successful. The surface modifications were all endorsed by FTIR. Now that the particles have been successfully synthesized and surface modified it will be possible to quantify nanoparticle uptake. It simply remains to culture the cells in a media containing the nanoparticles for a given period and measure the internalization of the nanoparticles using ICP. As a result of the successful modification with fluorescein uptake will also be visualized using fluorescent microscopy. Once this is completed it will open the door for other research using chemotherapy drugs grafted to nanoparticles as means to both target tumor cells and to kill them.

ACKNOWLEDGEMENTS

I would like to thank the National Science Foundation (NSF-EEC 9529161) which provided the funding, as well as the UWEB staff and faculty who provided their support.
FIGURE 5
(A) FTIR spectra of folic acid surface modified nanoparticles. (B) FTIR spectra of folic acid. (C) FTIR spectra of SAM amine coated nanoparticles. (D) FTIR spectra of native nanoparticles.

FIGURE 6
(A) FTIR spectra of methotrexate modified nanoparticles. (B) FTIR spectra of methotrexate. (C) FTIR spectra of SAM amine coated nanoparticles. (D) FTIR spectra of native nanoparticles.

FIGURE 7
(A) FTIR spectra of fluorescein coated to the folic acid surface modified nanoparticles. (B) FTIR spectra of fluorescein. (C) FTIR spectra of folic acid surface modified nanoparticles.

FIGURE 8
(A) FTIR spectra of fluorescein coated to the methotrexate surface modified nanoparticles. (B) FTIR spectra of fluorescein. (C) FTIR spectra of folic acid surface modified nanoparticles.
REFERENCES


The Influence of Environmental Conditions on the Encapsulation of HepG2 Liver Cells in Alginate

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Abstract: A study on the effects of alginate concentration, pH adjustment of encapsulation solutions, addition of medium components to encapsulation solutions, and geometry of the gels on encapsulated cell viability was performed using the HepG2 liver cell line. Bead encapsulation using non-adjusted solutions showed a maximum viability of 70% of the monolayer control and 95% growth over a 3 day period. Encapsulation with solutions adjusted to a physiologically favorable pH showed improved results with a viability that was maintained between 60 and 90% of the control and 100% growth over 5 days. To further improve viability, medium components were added to all encapsulation solutions resulting in 1000% growth and viability that was maintained at 90% of the control for 14 days. Gel geometry was found to have no effect on cell viability.

1. INTRODUCTION

Hydrogels are widely being investigated for mammalian cell immobilization for use in medicine and biotechnology. Applications of this technology include the possibility of creating a natural, new tissue or replacing or repairing lost or malfunctioning organs or tissues. Protein and nucleic acid drugs that cannot be taken in pill form also provide an incentive for studying new, implantable polymers for controlled drug delivery and gene therapy. In order for encapsulation to be a feasible option for treatment, however, more research is required in areas such as tissue supply, immune system rejection, oxygen transport limitations, and maintenance of cell viability and function [2]. Matrix materials used in mammalian cell encapsulation are often hydrogels that are crosslinked, hydrophilic polymers [7].

For successful encapsulation, several properties of the encapsulation materials must be optimized, including strength, transport properties, biocompatibility, ease of device formation, and gentle processing steps. The material needs to be robust so it will not degrade during the expected lifetime, but also biocompatible so as not to interfere with normal cell function. The material must allow sufficient diffusion and transport of oxygen, essential nutrients, and metabolic waste, and the material must also be sterilizable and able to be easily formed into desirable shapes. Harsh chemicals and temperatures cannot be used to form the encapsulation devices because capsules are made in the presence of living cells. Since an adequate amount of gases and nutrients need to reach the cells in order to maintain viability and functionality, the ability of the polymer to promote diffusion and transport in culture media and/or blood limit the size and shape used for encapsulation. Beads are often the geometry chosen because the high surface to volume ratio is ideal for minimizing diffusion limitations; thin film geometries have also been utilized [4, 7, 11]. Microencapsulation involves immobilizing cells in a thin membrane. The microcapsules are small (0.2 – 0.8 mm in diameter), and hold only a few hundred cells. Microcapsules are ideal for slow moving or stagnant media applications because transport limitations will not allow for larger geometries [2].

Alginate is a seaweed derivative that can be crosslinked with divalent calcium ions to form calcium alginate gels. The method of preparation allows alginate to be easily formed into many geometries. Alginate is sterilizable, and its hydrophilicity enhances its biocompatibility. Calcium alginate has successfully been used in many in vitro and in vivo applications including encapsulation of rat hepatocytes and islets (in vivo) and human hepatocytes and islets (in vitro) [1, 5, 7]. Recently, HepG2 cells encapsulated in alginate beads have been shown to express a range of liver-specific functions that approach those found in vivo [6, 9].

In our laboratory, calcium alginate encapsulated HepG2 cells are to become part of an in vitro system to study drug metabolism and drug interactions. Drug testing is primarily limited to two techniques – monolayer cell culture and animal testing, both of which have distinct disadvantages. Monolayer cell cultures can utilize human cells but do not
accurately mimic the three-dimensional nature of real tissues. Animal testing is unethical and uses nonhuman cells (e.g., rat or porcine systems) which interact differently with drugs than human cells. To solve this problem a cell culture analog system consisting of a multi-compartmental bioreactor for drug testing is being developed. Each compartment will contain different relevant cell types (e.g., hepatic, endothelial, etc.), with cell culture media flowing between them acting as a blood surrogate. The cells will be encapsulated at a high density (e.g., 1x10^6 cell/mL) to make them three-dimensional and more like real human tissues. There have been some successes with the development of cell culture analog systems [10, 3], but these in vitro models are not optimized for transport and maintenance of cellular function, and are therefore not readily applied.

Once a system is in place, the effect of drugs (both individual and in combination) on cellular metabolism, and the degradation of drugs by relevant cell types (e.g., liver) can be easily tested through this integrated reactor system. We are studying paclitaxel (Taxol®) as a model anti-cancer agent and have already characterized the response of monolayer cultures to clinically relevant concentrations [8]. The development of cell encapsulation technologies to better mimic the three-dimensional nature of human tissues is the next step in the development of an optimal in vitro system, and here we report on the encapsulation of HepG2 cells in calcium alginate.

2. MATERIALS AND METHODS

2.1 Cell Culture and Reagents

The human hepatic carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC). Cells were grown at 37°C in a 5% CO2 incubator in MEM supplemented with 10% fetal bovine serum (Atlanta Biologicals; Atlanta, GA). Sodium alginate, calcium chloride, sodium citrate, sodium chloride, D-glucose, HEPES buffer, and Trypan Blue dye were purchased from Sigma Chemical Company (St. Louis, MO).

2.2 Preparation of Encapsulation Solutions

Sodium hydroxide and hydrochloric acid solutions were used to adjust the pH of appropriate solutions and all solutions were 0.2 µm filter-sterilized. 2%, 1% and 0.75% (w/v) sodium alginate were prepared in dH2O. Solutions with medium components contained 1 g/L D-glucose and 2.35 g/L HEPES buffer. Calcium chloride (0.102 M) and sodium citrate (0.102 M) solutions were prepared in sodium chloride (0.15 M).

2.3 Spherical Cell Encapsulation

The monolayer cells were trypsinized, counted with a hemocytometer and centrifuged at 3600 RPM for 2 min. After centrifugation, medium was decanted and cells were resuspended in the appropriate sodium alginate solution. The cells were resuspended at concentrations of 1x10^6 cells per mL. The alginate/cell solution was transferred to a three mL syringe with a 30.5 gauge needle attached, and the sodium alginate/cell mixture was dropped into the calcium chloride solution, creating spherical capsules of approximately 2 mm in diameter. The beads remained in the stirred calcium chloride solution for 5 min to ensure gelation. Ten beads were placed into 16 mm wells to approximate the number of cells in the monolayer control (1x10^5 cells/well) and two mL of medium was added to immerse the beads. Medium was changed every other day, and beads and monolayer controls were sampled in approximately 24-h intervals.

2.4 Thin Film Encapsulation

The monolayer cells were trypsinized, counted with a hemocytometer and centrifuged at 3600 RPM for 2 minutes. After centrifugation, medium was decanted and cells were resuspended in the appropriate sodium alginate solution. The cells were resuspended at concentrations of 1x10^6 cells per mL. 200 µL of alginate/cell solution was added to an ethanol-sterilized coverslip inside of a six-well culture plate. Two mL of the calcium chloride solution were added to each well and five minutes was allowed for gelation. The calcium chloride solution was removed and five mL of culture medium were added to each well. Medium was changed every other day, and films and monolayer controls (seeded at 4x10^5 cells/well) were sampled in approximately 24-hour intervals.

2.5 Viability assay

Viability of the encapsulated cells was measured by dye exclusion. Two mL of the sodium citrate solution was added to each well containing cells encapsulated in calcium alginate. After the calcium alginate matrix had completely dissolved (approximately five minutes) the cells were collected by centrifugation (3600 RPM for two minutes), and resuspended in one mL fresh medium. One hundred µL of cell suspension was mixed with an equal volume of 0.4% Trypan Blue dye (in PBS), and 2 10 µL aliquots were counted using a hemacytometer. Viability percentage and number of viable cells were calculated by determining the proportion of cells excluding the Trypan Blue dye.

Monolayer cultures were detached with 10% trypsin (in HBSS), collected by centrifugation (3600 RPM for two minutes), and resuspended in one mL fresh medium.
µL of cell suspension was mixed with an equal volume of 0.4% Trypan Blue dye (in PBS), and 2 10 µL aliquots were counted using a hemacytometer. Each experimental treatment is a compilation of the results from a minimum of three different wells. Statistical relevance of data was determined using Tukey’s pairwise comparison at a confidence interval of 95%.

3. RESULTS AND DISCUSSION

3.1 Effect of Alginate Concentration on Encapsulated Cell Viability

Initial experiments were performed to determine the effect of alginate concentration on the viability of bead-encapsulated HepG2 cells (Fig. 1). Alginate and calcium chloride solutions were not adjusted to a physiologic pH of 7.2-7.4 and no additional medium components were added. In these experiments controls were monolayer cultures, which remained at greater than 85% viability throughout the course of the experiment. The viability of the encapsulated cells decreases sharply after the first 24 h to less than 50% of the control for all concentrations of alginate. For both 1% and 0.75% alginate encapsulated cells, viability remains essentially constant throughout the experiment (3 days), with a slight but significant rebound in viability observed for the 0.75% alginate encapsulated cells. For the 2% alginate encapsulated cells, the viability continued to decrease to less than 10% by the end of the experiment. In the 0.75% encapsulated cells 80% growth was seen when comparing the number of viable cells present on the first day with the number of viable cells present on the last day of the experiment. In the 1% alginate encapsulated cells there was 95% growth, and in the 2% alginate there was −50% growth, meaning that there were fewer viable HepG2 cells at the end of the experiment than at the beginning.

Successful encapsulation can be defined as retention of 90% viability as compared with control cultures. Because less than 70% viability was achieved in these experiments, it was determined that this is not an optimal method of encapsulation. The 2% alginate was omitted from future experiments due to the severe decrease in viability observed in initial experiments.

It is believed oxygen supply limitations are the most serious factor influencing cell viability and function [2], and the size of the beads (2 mm) could be inducing oxygen transport problems. Using systems to produce smaller alginate beads (~0.5 mm in diameter) has allowed successful mammalian cell encapsulation with 2% alginate concentrations [12]. This means that higher concentrations of alginate, which further limit transport in the beads, must be avoided. Other reasons for the decreased viability include unadjustment of the pH of the encapsulation solutions and potential osmotic and metabolic effects due to lack of inclusion of media components. The solutions were not adjusted to a physiologically favorable pH of 7.2-7.4, and the lack of glucose in the calcium alginate beads may induce cell lysis during encapsulation or starve the cells of glucose during the period of encapsulation, resulting in necrosis. The contribution of these factors was tested, and results are described below.

3.2 Effect of pH Adjustment of Encapsulation Solutions on Encapsulated Cell Viability

An experiment was performed to determine the effect of encapsulation solution pH on the viability of bead-encapsulated HepG2 cells (Fig. 2). In this experiment the HepG2 cells were encapsulated in 1% and 0.75% alginate using solutions adjusted to a physiologically favorable pH of 7.2-7.4 with no additional medium components added. The monolayer controls for this experiment were maintained at greater than 80% viability for the duration of the experiment (five days). The encapsulated cells in this experiment behaved in a very similar manner, showing no statistically significant difference between the viability of the cells encapsulated in 1% alginate when compared with the cells in 0.75% alginate. All encapsulated cells showed a distinct decrease in viability after the first day with a viability percentage of only 40% of the control. By the third day the cells in both alginate solutions had rebounded to within 90% of the control, and for the remainder of the experiment the cells re-
remained at a viability between 60 and 90% of the control. The liver cells encapsulated in 1% and 0.75% alginate showed a growth of approximately 100% over the course of the 5 day experiment. Although pH adjustment clearly improved encapsulated cell viability, there was a sharp decrease in viability observed after the first 24 H, and viability was not maintained at 90% of the control.

3.3 Effect of Addition of Medium Components to Encapsulation Solutions on Encapsulated Cell Viability

An extended time experiment (14 days) was performed with bead geometry using solutions that were pH-adjusted containing glucose and buffer of the same concentrations as culture medium. In addition, the calcium chloride and sodium citrate solutions were prepared in normal saline, which made the conditions of encapsulation closer to those found in vivo and further decreases the severity of the encapsulation procedure. This experiment was performed to determine the influence of osmotic and metabolic effects on encapsulated HepG2 viability. As can be seen in Figure 3, this experiment involved HepG2 cells encapsulated in unadjusted solutions, pH-adjusted solutions with no media components added, and pH-adjusted solutions with medium components added. The viability of the bead encapsulated cells in the adjusted solutions with medium components was maintained at ~90% of the control after the first day (monolayer cultures maintained ~85% viability). The cells encapsulated in the pH-adjusted solutions showed an initial decrease to 35% viability, but the cells in this treatment recovered by the second day to reach 90% of the control by day 10. The cells in the unadjusted solutions also showed an initial decrease to 30% of the control on the first day, and then begin a recovery on day 2; however recovery of cell viability was slower than that of the cells encapsulated in the pH adjusted solutions. The cells in the unadjusted solutions reach a maximum viability of 75% of the control on day 10, never reaching the viability percentage of the other two treatments. The adjusted solutions containing medium components shows a statistically significant difference in viability when compared with the viability of the pH-adjusted solution until day 6, and a significant difference when compared with the unadjusted solution until day 7. The pH-adjusted solution and unadjusted solution do not show a significant difference until day 14, when the pH-adjusted solution has significantly more viable cells than the unadjusted solution. All of the encapsulated cells showed approximately 1000% growth over the course of the 14-day experiment. This experiment also demonstrated that encapsulated cells can remain viable for extended periods of time in vitro.

3.4 Effect of Thin Film Geometry on Encapsulated Cell Viability

In an effort to decrease the size of the capsules, several experiments were performed using thin film geometry. As can be seen in Figure 4, these experiments involved HepG2 cells encapsulated in unadjusted solutions and pH adjusted solutions with medium components added. The HepG2 cells
encapsulated in a 1% alginate thin film with pH adjusted solutions containing medium components maintained a viability at approximately 90% of the control (monolayer control viability was greater than 90%) for the duration of the seven day experiment, a statistically significant difference from the cells encapsulated in unadjusted solutions. The cells encapsulated with unadjusted solutions maintained viability between 30 and 50% of the control until day 5 of the experiment when the viability began to rebound, finishing the experiment with a viability of 60% of the control. Over the 7 days of the experiment, the cells encapsulated in the adjusted solutions showed a growth of 500% while the cells in the unadjusted solution showed only 100% growth. There is no statistically significant difference in the growth and viability of the cells encapsulated in thin films when compared with the cells encapsulated in the bead geometry.

4. CONCLUSIONS

It was found that alginate concentration has a significant effect on encapsulated cell viability, with 0.75% and 1% sodium alginate solutions being the most successful at maintaining cell growth and functionality. It was hypothesized that 2% alginate trials were unsuccessful because of the transport limitations induced by the size of the beads and higher gel density. The adjustment of encapsulation solutions to a physiologically favorable pH of 7.2-7.4 increased the viability and growth of the encapsulated cells when compared with the viability in unadjusted solutions, and the addition of medium components further increased the growth and viability of the encapsulated HepG2 cells. It was found that altering the geometry of the alginate gels did not have a significant effect on encapsulated cell growth or viability. In adjusting these parameters, several alginate encapsulation techniques that retain liver cell viability at an acceptable level (approximately 90% of control) were developed. These results are significant not only because the viability of the liver cells is maintained at a high level, but also because inducing growth of encapsulated cells has been a challenge with other encapsulation methods [4], and significant growth was observed in our encapsulation systems. Ongoing experiments include varying cell seeding concentrations, continuing to optimize encapsulation procedures to maintain viability for extended time periods, utilizing a bead forming apparatus to create smaller beads, and encapsulating other relevant cell lines.

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REFERENCES


Complement Activation by PVA Hydrogels

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Abstract: Recent discoveries have shown that poly(vinyl alcohol) (PVA) complexes well with amino acids, especially glycine, arginine, and lysine. These complexes form a variety of materials, such as viscous liquids, gels, and films, and thus are useful for several medical applications, such as wound dressings and medical device gels. Due to its hydroxyl-rich surface, PVA activates the complement system, which in turn can cause inflammation. We postulate, that the OH groups of the PVA/AA hydrogels are “capped” through hydrogen bonding with the amino acids and therefore exhibit reduced complement system activation. The PVA/AA hydrogel films were incubated with human serum to induce complement activation. The serum was then analyzed for the amount of membrane attack complex or SC5b-9 produced. Compared to tissue culture polystyrene, PVA and PVA/AA gels were not large activators. In addition, infrared spectroscopy was used to determine hydrogen bond formation in the PVA/AA hydrogels.

1. INTRODUCTION

1.1 Complement System
Every year many implant operations are performed. Many of these operations fail because of encapsulation of the implant and inflammation caused by complement system activation [1]. Activation of the complement system is often caused by foreign objects and can occur via two pathways: either the classical (Fig. 1a) or alternative pathway (Fig. 1b). Both pathways are the result of enzymatic cascades and consist of two steps: initiation and amplification. The initiation step occurs spontaneously. The amplification step is a positive feedback loop in which the process of producing complexes is repeated. Both pathways produce the membrane attack complex (MAC), which is the primary cause of inflammation. The two pathways differ in that during initiation and amplification different complexes are formed [2, 3]. The complement system interaction is important with biomaterials, because inflammation may render implant useless and could even lead to death. Therefore, determination of the degree of complement system activation is essential when developing a new biomaterial.

1.2 Polyvinyl Alcohol
Poly(vinyl alcohol) (PVA) has been used since the 1920’s for many different purposes. PVA has been proven to be nontoxic and completely biodegradable and therefore could be used in the body [1]. Since vinyl alcohol does not exist in the natural state, PVA is made from vinyl acetate, by polymerization through a free radical reaction and subsequent
hydrolysis. PVA readily forms a hydrogel by inter- and intramolecular hydrogen bonding as shown in Figure 2. It has also been shown, that PVA will interact with other compounds, by either hydrogen bond formation or ionic interaction to form strong hydrogels.

In our lab, it has been discovered that PVA complexes rapidly with the amino acid glycine. Other amino acids have since been tested, and found that arginine, lysine, and arginine/glycine combinations also complex well with PVA. We postulate that when PVA complexes with the amino acids, the hydroxyl groups become “capped” through hydrogen bond formation with the carboxyl and amine groups of the amino acids.

PVA is known to activate the complement system, due to its hydroxyl rich surface. We believe that PVA-amino acid hydrogels will have reduced complement system activation, since the hydroxyl groups are “capped.” The reduced complement system activation should assist in the success of using these PVA-amino acid hydrogels as implants [4].

### 2. EXPERIMENTAL

#### 2.1 Materials

L-Lysine, (97%), L-arginine (98+%), glycine, (98%), and PVA (MW=85,000-146,000, 99% hydrolyzed) were purchased from Aldrich. All chemicals were used as received except for the PVA, which was purified as described below. Sodium chloride (certified ACS) was purchased from Fisher Scientific and used as a 9% aqueous solution. Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate was purchased from J.T. Baker and used as a 10% aqueous solution. The SC5b-9 (TCC) enzyme immunoassay kit was purchased from Quidel and used by following their instructions.

#### 2.2 Preparation of Gels

Polyvinyl alcohol was placed in a container filled with DI water to purify it and placed on a shaker for 24 h, changing the water at least once. The washed PVA was then gravity filtered, and washed with acetone and placed in the oven to dry.

An 8% PVA (w/v) solution was prepared by weighing out 0.48 g of washed PVA and adding 6 mL of sterile water. The amino acids concentrations (see Table I) were weighed out and the appropriate amount of sterile water was added. All solutions were placed in the autoclave (121°C, 45 min) to dissolve.

Once dissolved the amino acid solution was added to 8% PVA solution while on the vortex in 100 µl aliquots. Using a spatula the mixture was checked for gel formation after each addition, and gelled pieces were broken up to form a homogenous mixture while avoiding the formation of bubbles. Once completely added, 500 µl of PVA-Amino acid mixture was transferred into a well of a 24-well plate. These gels were dried at ambient condition covered by aluminum foil (4-5 days). Once dry, 1 ml of water was added to hydrate and swell the dried film. The day before analysis, 1 ml of 9% NaCl solution was added.

One experiment used freeze-thawed gels. These gels were made in the same way as before, except after casting in the 24-well plate, the entire plate was stored at 4°C for 18 h and then underwent four freeze-thaw cycles, which consisted of freezing the samples at –20°C for 1.5 h, with a

### Table 1: Amino Acid Concentrations

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Amt Gly (mg)</th>
<th>Amt Arg (mg)</th>
<th>Amt Lys (mg)</th>
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<td>1.2</td>
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<td></td>
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**FIGURE 2: PVA-BASED HYDROGEL STRUCTURES**

(a) PVA forms hydrogels by inter- and intramolecular hydrogen bonding. (b) In the PVA-Amino acid hydrogel it is believed that hydrogen bonding occurs when PVA complexes with amino acids. The hydrogen bonding can occur at both the NH\(^+\) and COO\(^-\) sites of the amino acid.
consequent thawing period at room temperature for 30 min. After the last thawing cycle, the plate was placed in the freezer over night. The amounts of amino acid concentration are shown in Table I.

2.3 Preparation of Serum
Whole blood was drawn from a healthy donor. It was allowed to clot for 1 h at room temperature, and an additional hour on ice. Serum was then poured off into centrifuge tubes, and centrifuged at 3500 rpm for 5 minutes. The obtained serum was aliquoted into polypropylene centrifuge tubes (7 mL each) and frozen at –70 °C until time of use.

2.4 Assay Analysis
On the day of analysis, 300µl of serum was added to each sample and incubated at 37°C for 90 min. After incubation, 10% EDTA solution was added to each sample to make a final concentration of 10 mM. The SC5b-9 assay was performed following the instructions from Quidel. The assay works by having precoated wells with mouse monoclonal antibody, which binds to the SC5b-9 in the samples. After incubation, wells are washed and conjugate is added, which binds to the SC5b-9 in the wells. A substrate to the antibody is then added to produce a colored product, which was quantified at 405 nm on an ELISA reader.

2.5 Infrared Spectroscopy
PVA and amino acid gels were made as described before. A thin layer of the PVA-Amino acid gel was placed on microporous ptytрафluorethylene (PTFE) Infrared (IR) Spectroscopy cards (3M) and dried for 24 hours under ambient conditions and subsequently dried under a vacuum for 15 hours. KBr pellets were made for glycine, arginine, and lysine, after pre-drying all reagents. IR spectra were obtained on a Brucker Vector 33 spectrophotometer as an average of 8 scans.

3. RESULTS

3.1 Complement System Activation
Figure 3 shows typical results of the amount of SC5b-9 complex produced through 90 mins activation at 37°C by the different materials. Serum serves as the negative control and the positive control was tissue culture polystyrene. The results are the average ± the standard deviation with n=4.

![Figure 3: Typical Results of Complement System Assay](image)

The serum was used as a negative control and the positive control was tissue culture polystyrene. The results are the average ± the standard deviation with n=4.

In Figure 4 the results for PV A/Arg/Gly hydrogels, prepared from different amino acid concentrations are shown. Only at the highest amount of amino acid concentration with 630 mg of glycine and 338 mg of arginine a significant difference occurs. Similar results were obtained with different concentrations of the glycine, arginine and lysine (see Table I). Results are not shown.

![Figure 4: Different Concentrations of Amino Acid](image)

Concentration of SC5b-9 for hydrogels prepared at different Gly/Arg concentrations are shown. The results are the average ± the standard deviation with n=4.

In Figure 5 compares freeze-thawed gels with air-dried gels prepared with the same amino acid concentration. Freeze-thawing did not significantly change the amount of complement activation with PVA/Gly gels. On the other hand, a significant difference in complement system activation was observed with PVA/Gly/Arg gels.

![Figure 5](image)

3.2 Infrared Spectroscopy
The IR spectra for glycine, PVA and PVA-Gly films are shown and compared in Figure 6. The O-H peak in the glycine spectrum is sharp and narrow, and in the PVA spec-
The O-H peak is broad. As can be seen, the O-H stretch is broader in the PVA-Gly spectrum than in the other spectra. The appearance of amine and O-H overtones and a new C=O peak in the PVA-Gly spectrum indicates hydrogen bonding.

Table 2 shows a summary of the IR modes which are mostly affected by hydrogen bonding, specifically the O-H, amine, and C=O stretch for glycine, arginine and lysine and PVA analogs. In all of the PVA-Amino acid gels, the O-H peak is broader, amine overtones appear, and the C=O peak has shifted.

4. DISCUSSION

Polyvinyl alcohol is known to activate the complement system because of the hydroxyl groups it contains [3]. We believe, hydrogen bonding occurs between PVA and amino acids and therefore “caps” the hydroxyl groups of the PVA, which reduces complement system activation. Several methods can be employed to determine complement activation, one such method consists of performing an assay for one of the several complement complexes which are formed during activation. Since it is not known by which pathway, the classical or alternative pathway, PVA activates the complement system, we decided to perform an assay which specifically quantifies the final SC5b-9 complex or MAC that is produced by both pathways.

For comparison, the baseline concentration of SC5b-9 in unactivated human serum had to be known and served as our negative control. Tissue culture polystyrene was used as a positive control because it is known to have high levels of complement system activation. Throughout the experiments, unactivated serum and tissue culture polystyrene were used as our standards.

Even though PVA is a complement system activator, the results shown in Figure 3 indicate that PVA only minimally activates the complement system compared to tissue culture polystyrene. The amount of SC5b-9 complex produced by PVA was similar to quoted values in the literature. According to Sefton et al., the amount of SC5b-9 detected was 2500 ng/mL for PVA gels [5]. This value is comparable to our value obtained, which was 1700 ± 300 ng/mL (after subtraction of the baseline serum level). Hydrogels obtained from PVA and amino acid mixtures showed the same levels of SC5b-9 as unactivated serum, therefore no activation of the complement system had occurred. These results are consistent with our theory “capping” of the O-H groups of the PVA by hydrogen bonding with the amino acids.

In Figure 4, we compare a variety of amino acid concentrations. By changing the amount of amino acids added to PVA, we were hoping to see a correlation of the degree of hydrogen bonding. However, we found complement activation did not significantly change, except with the highest amounts of amino acids concentrations of PVA, arginine and glycine. One possible explanation for this could be that the low amino acid concentrations were enough to “cap” the hydroxyl groups of the PVA, and if any excess amino acid was present it was washed away during hydration. However, with the larger amounts of amino acid there might be another complex being formed that does not allow for excess amino acid to be washed away. The excess amino acid introduces COO⁻ and NH₃⁺ groups, which causes complement system activation due to the charge. These extra charges in the gel could lead to complement system activation. This theory still requires additional investigation to be proven.
In Figure 5, we compared the effects of freeze-thaw-drying to air-dried gels. It was believed freeze-thawing the gels would reduce the amount of complement system activation. Freeze-thawing causes a more highly crystalline form of PVA and enables all of the O-H groups of PVA to become “capped.” By freeze-thawing, hydrogen bond formation is induced [6]. However, there was no significant change in the amount of complement system activation between the freeze-thawed samples and the air-dried samples. The reason for this could be due to how hydrogen bonds form differently in freeze-thawing and air-dried gels, or even freeze-thawing incorporates all of the amino acid so excess amino acid is unable to be washed away during hydration, leaving excess O-H and NH$_3^+$ groups to cause complement system activation. This theory still needs to be tested.

Infrared spectroscopy was used to determine if hydrogen bonding between the PVA and amino acids occurred and therefore led to “capping” hydroxyl groups of PVA. IR spectroscopy is sensitive to chemical changes which is apparent by changes in the spectrum. For example, a “free” O-H peak is usually observed around 1720-1706 cm$^{-1}$ as a sharp peak. However, when hydrogen bonding occurs, the peak becomes broader and the “free” peak disappears. This effect can be seen when we compare glycine film with PVA/glycine films (Fig. 6), indicating hydrogen bonding. In addition, a broadening of the O-H stretches of the PVA-amino acid films is seen when compared to the O-H peaks of just the amino acid (Table II). A second stretch affected by hydrogen bonding is the C=O; the C=O peak of a carboxylic acid usually occurs near 3520 cm$^{-1}$, which represents a “free” C=O peak. When the carboxylic acid is involved in hydrogen bonding, the peak is shifted to an absorption higher than 1600 cm$^{-1}$. The results for C=O are shown in Table 2 and in general, indicate hydrogen bonding of the COO$^-$ of the amino acids with PVA. This is absorption is observed in Table II. Primary amines, such as the amine groups in amino acids, have absorptions between 3500 cm$^{-1}$ and 3400 cm$^{-1}$. These are representative of “free” amines. When hydrogen bonding occurs, amine overtones occur. The same is true for hydroxyl groups. In all of the PVA-Amino acid films overtones appeared (Table II) [7]. Through the IR data obtained, we were able to realize hydrogen bonding does occur, which is consistent with our theory.

### Table 2: IR Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>OH Stretch</th>
<th>Overtones</th>
<th>NH$_3^+$</th>
<th>C=O (free)</th>
<th>C=O (H-bonded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVA</td>
<td>3650-2900</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gly</td>
<td>3350-2100</td>
<td>-</td>
<td>-</td>
<td>1677</td>
<td>-</td>
</tr>
<tr>
<td>Arg</td>
<td>3500-2750</td>
<td>-</td>
<td>-</td>
<td>1669</td>
<td>-</td>
</tr>
<tr>
<td>Lys</td>
<td>3600-2350</td>
<td>-</td>
<td>2145</td>
<td>-</td>
<td>1585</td>
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<tr>
<td>PVA/Arg</td>
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<td>-</td>
<td>1676</td>
<td>1632</td>
</tr>
<tr>
<td>PVA/Gly</td>
<td>3650-2000</td>
<td>+</td>
<td>2150</td>
<td>-</td>
<td>1618 (br)</td>
</tr>
<tr>
<td>PVA/Lys</td>
<td>3650-2000</td>
<td>+</td>
<td>2126</td>
<td>-</td>
<td>1562 (br)</td>
</tr>
</tbody>
</table>

### 5. CONCLUSION

Activation of the complement system causes inflammation. PVA is considered an activator of the complement system. However, once combined with amino acids, the PVA hydrogels do not significantly activate the complement system when compared to serum as seen by the levels of SC5b-9 produced. The reason for this difference is that the O-H groups of the PVA become “capped” due to hydrogen bonding with the amino acid as shown by IR. This hydrogen bonding takes place at both the NH$_3^+$ and COO$^-$ sites of the amino acid. Since we were able to show that complement system activation does not occur, these materials can now be tested for biocompatibility.

### ACKNOWLEDGEMENTS

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Drug Release from Hydroxyethyl Acrylate Hydrogels

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Abstract: Drug release from poly(hydroxyethyl acrylate) hydrogels was tested using different combinations of hydrophobic and hydrophilic cross-linkers and model drugs. Hydrogels, containing the drug of interest, were polymerized using free radical initiators. Drug release was tested in vitro by measuring diffusion of the drug into phosphate buffered saline solution at a pH of 7.4. A significantly higher amount of the hydrophobic drug diffused out of the polymer when the gel was prepared with the more hydrophobic crosslinker, as determined by UV spectrophotometry. The hydrophilicity of the crosslinker did not significantly affect the release of the hydrophilic drug. Additionally, we showed that the release of the hydrophobic drug was greater than that of the hydrophilic drug in all cases.

1. INTRODUCTION

Most hydrogels are highly biocompatible, which makes them an extraordinary and important basis for research [1]. A hydrogel is a hydrophilic network of polymer that is held together by covalent cross-linked bonds[2]. Hydrogels swell and may absorb water ranging from 10%, up to thousands of times their dry weight[3]. The fact that hydrogels swell in water makes them a unique source for drug delivery. As the polymer network swells, the drug is released, either by diffusion through the water in the matrix or through the polymer matrix itself. Hydrogels can be used for both local and systemic drug delivery[4]. The purpose of this research is to investigate the release of both hydrophilic and hydrophobic drugs from poly(hydroxyethyl acrylate) (p(HEA)) hydrogels with various cross-linkers. Two different cross-linkers, tetraethylene glycol dimethacrylate (TEGDMA) a hydrophilic molecule, and 1, 4 – butanediol diacrylate, a more hydrophobic molecule, were used to crosslink the HEA. The molecular structures of the monomer and the two cross-linkers are shown in Figure 1.

Two different model drugs, methylene blue, which is hydrophilic, and 4’, 5’ – dibromofluorescein, which is hydrophobic, were added to the hydrogel mixtures. The molecular structures of the model drugs are shown in Figure 2.

![Figure 1](image1.png)

**Figure 1**
Molecular structure of monomer and cross-linkers: (a) hydroxyethyl acrylate, (b) tetraethylene glycol dimethacrylate, (c) 1,4 - butanediol diacrylate.

![Figure 2](image2.png)

**Figure 2**
Molecular structures of model drugs: (a) methylene blue, (b) 4’,5’ - dibromofluorescein.
The hydrogels are polymerized using free-radical initiators, which cause the formation of a covalent bond between the vinyl groups in the monomer and in the cross-linker. Because the cross-linkers have two vinyl groups, this causes the polymer to form into a hydrogel network. Figure 3 shows a schematic drawing of what such a network would look like.

![Schematic of hydrogel network.](image)

The drug is incorporated into the hydrogel by mixing it into the solution before the hydrogel polymerizes. This causes the drug to be dispersed within the hydrogel network.

2. Experimental

2.1 Materials

All hydrogel samples were prepared with hydroxyethyl acrylate, (Polysciences). The solvents used were deionized water, ethylene glycol (Fisher), and ethanol (Pharmco). Ammonium persulfate (NH₄)₂S₂O₈ and sodium metabisulfite Na₂S₂O₅ were used as the free radical initiators (both from Aldrich). Two different cross-linkers, tetraethylene glycol dimethacrylate, (TEGDMA), and 1, 4 – butanediol diacrylate were used (both from Polysciences). Methylene blue and 4', 5' – dibromofluorescein were used as model drugs (both from Aldrich).

2.2 Methods

Hydrogels were mixed according to the amounts shown in Table I. First the solvents were mixed together. Then 5.0 mL HEA was added to each sample. Next, the respective cross-linker and model drug were added. The two initiator solutions were mixed together and then added to the hydrogel solutions. Between each step, the solution was mixed well on a vortexer.

Hydrogel solutions were injected between 4” x 6” glass plates separated by two 0.03-inch spacers. The solutions polymerized overnight. After soaking the glass plates containing the gel in water for 1 h, the plates were separated, and sample disks were cut from the hydrogels, 17/32 inches in diameter. These were placed into 20 mL vials with 5.0 mL of phosphate buffered saline solution (PBS), pH 7.4. Five samples of each of the four different gels were used. The vials were placed in a rack on a shaker. At designated time points, the samples were removed from the shaker, the elution media was removed, the samples were rinsed in PBS, 5.0 mL of fresh PBS was added to each vial, and the samples were placed back on the shaker.

The UV absorption of the extracted samples of elution media were measured by UV spectrophotometry on the Versa Max tunable microplate reader, at wavelengths of 609 nm and 502 nm for methylene blue and 4’, 5’ – dibromofluorescein, respectively.

2.3 UV Spectrophotometry

In order to obtain UV spectra of the model drugs, solutions of methylene blue (0.0002% in PBS), and 4’, 5’ – dibromofluorescein (0.001% in PBS), were measured on a HP 8452A diode array spectrophotometer. Spectra are shown below in Figure 4a and 4b. Standard curves were prepared using methylene blue concentrations ranging from 0.000 to 0.002 mg/ml and 4’,5’ – dibromofluorescein concentrations ranging from 0.00 to 0.01 mg/ml. Methylene blue samples were read at a wavelength of 609 nm and 4’,5’ – dibromofluorescein samples were read at 502 nm, both on the Versa Max tunable microplate reader. Standard curves are shown in Figure 4c and 4d.

![UV Spectrophotometry graphs.](image)
3. RESULTS

Figure 5 shows the amount of model drug released from the HEA hydrogels with respect to time.

As shown in Figure 5, the hydrophobic model drug, 4’, 5’ – dibromofluorescein exhibited a greater release from the hydrogel than the hydrophilic model drug, methylene blue. The different cross-linkers did not appear to make any difference in the release of the methylene blue samples. The model drug, 4’, 5’ – dibromofluorescein showed a greater release of the hydrophobic drug with the hydrophobic crosslinker, 1, 4 – butanediol diacrylate.

4. CONCLUSION

In this study, we showed that release of a hydrophobic drug from an HEA hydrogel is affected by the hydrophilicity of the crosslinker. More of the hydrophobic drug, 4’, 5’ – dibromofluorescein diffused out of the polymer when the gel was made with a more hydrophobic crosslinker, 1, 4 – butanediol diacrylate. We also showed that the hydrophilicity of the crosslinker did not significantly affect the release of the hydrophilic drug, methylene blue. Additionally, we showed that the release of the hydrophobic drug was greater than that of the hydrophilic drug in all cases.

In general, drug release from a hydrogel occurs either by diffusion through the water in the matrix, or diffusion through the matrix itself. Possible complicating factors in the experiment include an excess of cross-linker, monomer, initiator, or solvent remaining in the hydrogels, diffusing out with the drug. This may have resulted in discrepancies in the UV spectrophotometer readings and definitely would affect the way the drug interacted with the hydrogel and therefore, diffused out of the hydrogel. There may also be possible ionic charge interactions between the drug and the hydrogel. Additionally, the drug was in the hydrogel when it was polymerized, which could possibly result in the drug being linked into the polymer matrix covalently or ionically, causing phase separation, or pore size issues. Any drug that may have been attached to the matrix, would not have been released. A difference in pore size would affect the ability of the model drug to be released either more quickly or more slowly. All of these factors could affect the way in which the drug was released from the hydrogel. It would be beneficial to do further studies to incorporate the drug into the hydrogel after polymerization and test the release in that manner.

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1. INTRODUCTION

One of the major obstacles in today’s biomedical industry is the natural response of the human body to effectively ‘wall off’ foreign objects with a layer of tissue. The consequences of this include a reduced efficiency of the implant and strain on the body in maintaining this ‘wall.’ One possible antidote is to develop biomaterials that closely mimic the biological model at the molecular level. Admittedly, we don’t have to reproduce the living model in its entirety. We are simply interested in the outermost (surface) layer insofar as its impact on the interaction between living system and biomaterial. To do this, we need a method of ‘seeing’ what exactly is on this surface. That is, the molecular composition of the surface.

What comprises a surface and why is it so important? It is claimed by some that the surface can be described as a unique state of matter [1] – one with characteristics that hold it apart from gases, solids, and liquids. Generally, it is accepted that a ‘surface’ includes the first 100 Å [2]. Because the surrounding environment of surface molecules differs from that of bulk molecules (Fig. 1), surface atoms have higher energy than their bulk counterparts (due to unfilled valences, uneven distribution of attractive/repulsive forces, etc.) [3]. Due to contact with other materials and different phases, the surface layer is also prone to react with these other materials (e.g. the outermost layer of most aluminum products is actually aluminum oxide). The key in understanding the molecular composition of a surface is to be able to analyze this region without interference from the bulk structure – surface analysis.

One issue that arises is that the surface makes up a comparatively small percentage of the entire structure. This makes it imperative that the analysis technique uses samples from the surface regions. Additionally, the molecules of interest will exist in much lower concentrations than normally is the case in bulk studies. Thus, surface analysis requires a highly sensitive analytical technique. Since the final goal is to look at samples drawn from living systems (proteins, lipids, etc.) we are dealing with an extremely wide mass range. Note that we are interested in entire molecules – to correctly identify the composition of the surface, we must attempt to detect the entire molecule each time.

With a theoretically unlimited mass detection range (perfect for high mass proteins), and parts per billion sensitivity, Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) is the ideal method for surface studies. ToF-SIMS works by sending a charged particle (primary ion) towards the surface. Upon impact, the energy associated with this primary ion is transferred to the surface allowing part of this surface to break free (secondary particles). A few of these secondary particles are charged (ions), all of which are accelerated to the same kinetic energy and their velocity is measured. Ions of higher mass will exhibit lower velocities than smaller ions at the same kinetic energy. All of this is performed under vacuum conditions. Of course, there are various factors that have influence over the efficiency of this process - the angle at which the original charged particles hit the surface, the time lag between successive firings of the primary ions, and the area over which the primary ions strike the surface. All samples in this study apply the principles of static SIMS. That is, to keep the ion bombardment so low that on average only one primary ion will strike a local region. The tolerance for this is generally accepted to be 1 x 10¹² ions/cm² [2]. In addition to these manipulations, changes in the preparation techniques of the samples themselves will have a significant impact on the accuracy and sensitivity inherent in the data. This study is focused on the effects of various sample preparation techniques and thus instrument variables are kept constant when possible.

As with all data collection techniques, the goal is towards increasing sensitivity. In developing alternative preparation techniques for the actual sample, we can look to similar surface analysis techniques, specifically, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). This technique is remarkably analogous to ToF-SIMS, with one major difference being the use of a laser as the
source instead of a charged particle (see above for discussion). Additionally, instead of simply applying the sample biomolecules neat onto the substrate (as in traditional ToF-SIMS preparation methods), the sample preparation calls for a mixture of the biomolecules with a matrix. It is theorized that the matrix ‘softens’ the impact and minimizes the damaging effects on the molecular ions – resulting in less fragmentation and thus, a higher number of observed molecular ions. This study applies the MALDI-MS preparatory technique of using matrix molecules while retaining the traditional ToF-SIMS approach to instrumentation, using Cs⁺ as the primary ion.

2. EXPERIMENTAL

Proteins used in this experiment were Angiotensin II (1046), Fragments 1-7 Angiotensin (899.0), Ala-Pro-Gly [Ile³Val⁵] Angiotensin (1270), Asn¹Val⁵ Angiotensin (1031), and Substance P (1347.7). All were obtained from the Sigma-Aldrich Company. Protein solutions were prepared at a concentration of 0.01mg/ml in MilliQ water. Sinapinic acid (SA) and 2,5-dihydroxybenzenoic acid (DHB) solutions were prepared at concentrations of 20mg/ml and 15 mg/ml, respectively. Protein and matrix solutions were mixed 50:50 prior to placement on gold or silicon substrates in 1 ml droplets. Each droplet contains 0.005 mg of protein. Unused protein solutions were stored at < 0°C.

In desalted samples, the matrix solution was passed through a cation exchange desalting column twice prior to mixture with the protein. Silicon wafers were sonicated three times in a mixture of acetonitrile, methyl chloride and methanol then dried under nitrogen. Gold wafers consisted of a 1000 Å layer of gold over a silicon wafer.

All samples were analyzed using a Physical Electronics Model 7200 Time-of-Flight Secondary Ionization Mass Spectrometer. Primary ions were Cs⁺ in all cases, pulsed at 3 kHz. Silicon substrates were run as insulators, using a low-energy pulsed electron gun to neutralize surface charging. Gold substrates were run as conductors, without surface neutralization. Criteria for static SIMS were met, with a total ion dosage of 10¹² ions/cm². Data acquisition and instrument control were performed using PHI COMPASS software on a Sun workstation. The PHI TOFPak software was used in analysis of the SIMS data on Pentium PCs.

3. RESULTS AND DISCUSSION

3.1 Angiotensin II

Neat Angiotensin II prepared on silicon and gold substrates yielded no molecular ions (Fig. 2). In both cases, the highest peak was identified as Na⁺ (23). With the addition of DHB, the sample on silicon yielded a relatively large quantity of molecular ion, enough so that the isotopic distribution is clearly visible. The amount of M⁺ detected makes it visible even when looking at the entire sampling mass range. This sample also exhibits a high matrix ion signal, which is expected in this preparation technique. One possible explanation of how matrix enhancement works suggests that molecular ions come from molecules that either have been incorporated into matrix crystals or are sitting directly on top of the matrix crystal. As with the neat preparation, we see a high concentration of salt (Fig. 3a). On the gold substrate, there is no molecular ion evident. Inspection of the matrix ion reveals a much lower concentration than in the silicon treatment. This further supports the theory requiring that matrix and analyte be physically connected, not merely in the immediate vicinity. Again, the spectra shows evidence of high salt content (Fig. 3b). Preparations involving sinapinic acid and Angiotensin II yielded similar results. Looking at the silicon substrate we see a molecular ion peak resolved enough to distinguish the isotopic pattern, although this peak is at a considerably lower concentration than when paired with DHB. Checking the matrix ions we can see that they were actually detected at higher concentrations than in the preparations involving DHB. However, comparison reveals that, in the sinapinic acid preparation, the salt peak was an order of magnitude higher than in the DHB treatment (Fig. 4a). When prepared on gold with sinapinic acid, there is no molecular ion detected from Angiotensin II, despite having a very strong matrix ion. Again, the salt peak is much higher than the matrix peaks.
It is theorized that the presence of any significant amount of sodium ions will suppress the production of molecular ions, promoting fragmentation. To test this, the same samples were prepared as above, only with desalted matrices. Concerning Angiotensin II and desalted DHB, we find evidence of the molecular ion, with enough resolution to see the isotopic pattern. However, we note that the concentration of the molecular ion in this sample is significantly lower than in its salt-containing version. What distinguishes this sample from previous ones is the relatively low concentration of sodium ions (Fig. 3c). On gold, desalting is the first treatment that has yielded molecular ions. While the isotopic pattern is visible, it is at a much lower concentration than in samples containing silicon. Again, the salt content is quite low, with a relatively high matrix ion content (Fig. 3d). Looking at samples including desalted sinapinic acid, we can see that on silicon we do get a molecular ion peak. However, despite relatively high matrix ion peaks and low sodium content, the molecular ion is detected at the lowest concentration of the silicon samples, with its isotopic pattern barely detectable (Fig. 4b). In the sample containing Angiotensin II and desalted sinapinic acid, a molecular ion was barely detectable – the isotopic patterns were not clear. However, the sodium ion was present in quantities an order of magnitude higher than matrix concentration, which was relatively high.

3.2 Substance P

Looking at a biomolecule of higher mass than Angiotensin II, we prepared neat samples of Substance P on both substrates and were unable to detect the protein. As with Angiotensin II, we were unable to detect molecular ions on the silicon substrate, despite the nonexistent sodium peak. On gold, a very faint molecular ion peak was detected, along with a medium sized sodium peak. In samples containing DHB on the silicon substrate, we can see a small molecular ion peak, indicating low concentration. There is also a very high sodium content and relatively high concentration of matrix ions. On the gold substrate with DHB, we can see a vague peak where the molecular ion should be, but at an extremely low count. The sodium ion is very high while matrix ions are also quite high. With the matrix molecule sinapinic acid, we are unable to detect the molecular ion on silicon. The sodium peak is not extremely high and matrix concentration seems to be buried in background ‘noise.’ On gold, there is an identifiable molecular ion peak. However, the matrix ions are detected in such low amounts that the peaks blend into the background ‘noise’ of the fragments. Additionally, the sodium signal is almost nonexistent.

We again obtained spectra from the desalted analogs of the samples described above to elucidate what effect the presence of the sodium ion has on molecular ion formation. For Substance P and desalted DHB on silicon we saw a small peak indicating the molecular ion, at a count level that is just under what was obtained for the salt-containing version. Additionally, despite the desalting techniques, there was still a relatively high concentration of sodium ions. Matrix ions were also present in relatively large quantities. When on the gold substrate, the molecular ion was detected at roughly the same levels. Sodium concentrations were relatively high, with the highest peaks belonging to hydrocarbon fragments at m/z = 41 (C3H5). Matrix ions were detected at relatively high concentrations. Looking at Substance P with desalted sinapinic acid on the silicon substrate, we see that no molecular ion was detected. On the gold substrate, however, we did see a molecular ion with a small peak. The sodium ion was detected at levels roughly two orders of magnitudes more than the matrix ions.
3.3 Substance P and Angiotensin II

For ToF-SIMS to be a viable analysis tool to aid in identifying unknown biomolecules in biomaterials development or quality control, it must be useful in an environment where there are multiple molecules present. To model this we have applied both Substance P and Angiotensin II to both substrates with the various matrices. For the samples containing DHB on silicon, we see only a hint of the Angiotensin II molecular ion and no sign of the Substance P molecular ion, however there is a very high salt content and a significant detection of matrix ions. In the presence of DHB on gold, we easily see both molecular ion peaks, with their isotopic distributions. Matrix detection levels are relatively high, as is the salt content.

For the samples with desalted DHB matrices on silicon we detect both molecular ions at levels approximately the same as in the salt-containing versions. Salt levels are high while matrix ions are detected at low levels. The same sample on gold reveals that we see both molecular ions and the resolution clear enough that we can view the isotopic pattern for the Angiotensin II molecular ion. The salt content is relatively high while matrix ions are also detected at relatively high levels.

3.4 Fragment 1-7 Angiotensin

Treatments consisting of DHB and Fragment 1-7 of Angiotensin II on both substrates yielded no molecular ions. Additionally, both samples had sodium as the most abundant ion. Both also have strong matrix ion peaks.

Again, using desalted matricies returned different results than their salt-containing counterparts. In this case, desalted DHB and the protein on silicon yielded a molecular ion, but too low in intensity to resolve the isotopic pattern. Despite the desalting process, salt content was high. Matrix peaks were also high. Desalted DHB on gold resulted in a molecular ion peak in which the isotopic distribution could be clearly noted. The salt peak was clearly subdued, however, matrix peaks blended into the background.

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4. CONCLUSIONS

In every case presented above, inclusion of matrix molecules in the sample preparation correlated with increased molecular ion detection. The complete explanation of how and why the matrix affects molecular ion fragmentation and formation is as yet unclear. However, this study has shown that, in twenty-two matrix/protein/substrate combinations, there was not one case in which less molecular ion was detected than in the neat protein treatment. Thus, it can be said that with regard to the proteins and matrices discussed herein, the practice of including a matrix in sample preparation techniques is a viable method in the quest for higher molecular ion detection.

In the above eleven comparisons, seven of these showed that desalting the matrix correlated with an increase amount of molecular ion. Of these, only two – Substance P and Angiotensin II with DHB, Fragment 1-7 Angiotensin with DHB - were on a silicon substrate. The remaining six were on gold substrates. Additionally, two samples were prepared with sinapinic acid as its matrix – Angiotensin on gold and Substance P on gold. Fragment 1-7 Angiotensin with DHB on both silicon and gold experienced increased molecular ion detection when desalted matrices were employed. Angiotensin II in combination with either matrix on gold fell into this category as did both matrices with Substance P on gold.

Of the four treatments that suffered a drop in molecular ion count with the use of desalted matrices, only one – Substance P and Angiotensin II with DHB was on gold. Angiotensin II and sinapinic acid was the only treatment in this grouping prepared on a substrate of silicon. Both matrices with Angiotensin II on silicon also fall into this category.

Given the above summary, it is tempting to say that the correlation (or lack thereof) between molecular ion detection and the practice of desalting matrices seems to depend on the protein/substrate combination. We can argue that in some cases the desalting of the matrix does correlate with increased molecular detection and it too can be considered a viable method to detect a larger amount of molecular ions from a given sample concentration.

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