Protein Induced Adhesion and Release of Human Umbilical Vein Endothelial Cells

Katherine Hauwiller

Introduction

The ability to adhere a unique type of cell has many medical applications and is at the core of a wide variety of medicinal research and developments. Processes ranging from treatment of Atherosclerosis to growth of new capillaries revolve around the basic adhesion of cells. [1, 2]

Because of the many applications, various methods have been developed for selective adhesion of cells. For example, radical flow chambers were modified with RGD peptide to HUVECs.[3] However, these methods have not accomplished both selective adhesion and subsequent release of cells.

In this study, we proposed a new material design to selectively allow cells to adhere and subsequently release cells. For this experiment, HUVECs were used as a model cell type and CysALDV was used as the adhesive protein for HUVECs. The inside of a microfluidic channel was modified with CysALDV, and then seeded with either HUVECs or 3T3 cells. The channels were then washed to flush the cells that did not adhere from the channel. Our hypothesis was that the HUVECs in the CysALDV channel would adhere, but all other combinations would not. Following the adhesion of the HUVECs, a molecular trigger B-PEG was added to release HUVECs.[4] The B-PEG will be suspended in solution, then pumped through the channel in which the HUVECs are encapsulated. It was hypothesized that the HUVECs should be released from the CysALDV.

Materials and Methods

Protein Expression: CysALDV

1L of 2xYT medium was prepared in 3 individual 2L flasks. The flasks were covered with aluminum foil and autoclaved on liquid cycle. One colony of Escherichia coli (E. coli) was cultured overnight in 60mL of 2xYT medium containing 120µL Ampicillin and 150µL Kanamycin.

2mL of Ampicillin and 2.5mL of Kanamycin was added to each liter of 2xTY media. The culture was grown until the OD600 was approximately 0.7. 1mL was taken from a flask as a pre-induction control and was stored at 4˚C. 1mL of IPTG was added to each flask to induce protein expression. The cultures were incubated at 37˚C for an additional 4 hours.

To separate the cells from the media, the culture was centrifuged in a SLA 1500 rotor at 5,000 rpm for 10 minutes. The pellet was resuspended in 100mL 8M urea at pH 8 and stored at -80˚C overnight.

Cells were thawed in a shaker incubator at 37˚C until most ice was melted. The sample was centrifuged at 12000 rpm for 20 minutes using a SLA 1500 rotor to separate the cell debris from the protein. The pellet was discarded and the supernatant was again centrifuged at 12000 rpm for 20 minutes. The

resulting supernatant was sonicated 6 times for 10 seconds, swirling the bottle in between sonications to prevent protein degradation due to overheating.

The protein solution was then poured into a nickel column for purification. A series of 8M urea washes starting with pH 8 followed by pH 6.3, 5.9 and 4.5 were used to elude the protein from the column.

Protein Purity Analysis

The protein solutions acquired from the 8M urea washes were run on an SDS gel for purity analysis. 12µL of the protein solution was mixed with 3µL of dye and 14µL of the product of each wash was pipetted into individual channels.

Note: The expression and purity analysis of CysA and BCys only differed in the bacteria colony chosen.

Production of PDMS channel

Silicone Elastomer Base was mixed with Silicone Elastomer Curing Agent at a 10:1 ratio and was poured over a mold. Bubbles were removed by placing it in a vacuum overnight and crosslinking was enhanced the following day by applying heat at 37˚C for 4 hours. The channel was removed from the mold and cut leaving 1.5cm of excess PDMS around the channel inlet for future bonding to glass surface. Holes were punctured on both sides of the inlet with the blunt needle. These holes were used for pumping solutions through the channel. See Figure 1 on the following page.

B-PEG Production

20mg of Bcys was dissolved in an 800µL solution of 20M TCEP in PBS. The pH was adjusted to 4.5 and the sample reacted for 1hour at room temperature. 800µL of PBS was added to dilute the concentration of TCEP and the pH was adjusted to 7.0. 200mg of 10k PEG-maleimide was dissolved in 2mL of PBS. One fifth of this solution was added to the protein solution every 30 minutes until all was added. The reaction was allowed to continue overnight at room temperature.

The pH of the overnight reaction was adjusted to 8.0 and was loaded on a Ni-NTA column to remove the unreacted PEG-maleimide. The column was washed with 8M urea with a volume 10 times that of the resin. The B-PEG and Bcys were then eluted from the column using pH 4.5 8M urea. The pH of the eluted fraction was adjusted to 7.5 and was placed into dialysis membranes to begin the process for volume reduction. Dialysis was done for 4 days, changing the water every 2 hours, five times a day. On the fifth day the sample was froze and placed on a lyophilizer for three days. The resulting dry mixture of B-PEG and Bcys was then dissolved in 1mL of binding buffer (100mM Tris-HCL, 500mM NaCl, 1mM EDTA, 8M urea, pH 7.5). 5mM of TCEP was added to the solution and was allowed to react for 1 hour at room temperature. The pH was then adjusted to 7.5 and 1mM of EDTA was added to the solution. The sample was diluted with 1.5mL of binding buffer.

The Bcys was then removed using a Thio Sepharose 6B resin. The sample was loaded on to the column and the flow rate was adjusted to about 0.42mL/minute. The column was washed with 2 times the volume of the resin. The flow through from these two contained the B-PEG. The urea was then

removed from the sample through 3 days of dialysis and was freeze binding buffer dried on the lyophilizer for a week..

B-PEG Purity Analysis

A small sample of B-PEG solution was concentrated using a 3x Centricon centrifugal filter device. 12µL of the original B-PEG, the concentrated B-PEG and a BCys solution were placed in separate tubes and each was mixed with 3µL of dye. These tubes were capped and boiled, then run on a SDS gel.

Cleaning Glass Slides

Glass slides were soaked in 10mL of H2O2 and 15mL of H2SO4 for 2 hours. After 2 hours, slides were rinsed with distilled water for 1.5 hours. Glass was blown dry with Argon gas.

Glass surface modification: Pre-Channel Assembly

Glass slides were placed in a UV Ozone generator for 8 minutes. 20µL of (3-Aminopropyl)triethoxysailane (APTS) was pipetted across the middle of the glass slide where the inlet of the PDMS channel will be placed. After 30 minutes the APTS was washed off with distilled water and the slides were incubated for 1 hour at 80˚C.

Glass Surface and PDMS Adhesion

The glass slide and PDMS were placed in a UV plasma cleaner for 1 minute to prepare the surfaces for bonding. PDMS was placed on the modified side of the glass surface immediately after removal from the UV cleaner as seen in Figure 1.

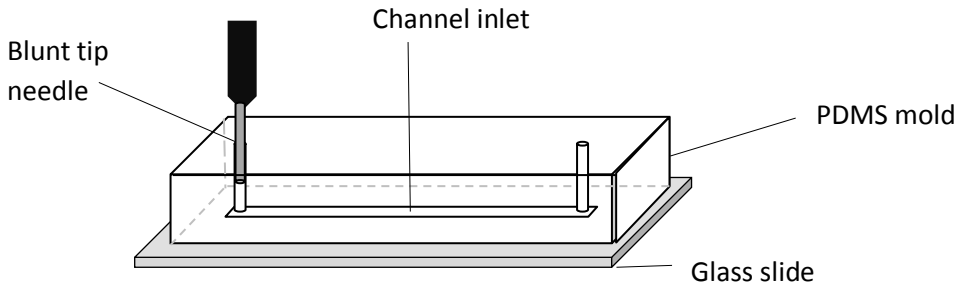

Figure 1: The assembled channel
Solutions are pumped into the channel via the blunt tip needle and exit the channel via the hole on the other side of the channel. The channel is 5cm long. Width is either 1 or 2mm and depth for the first trial was 76µm and 65µm for the second.

Glass Surface Modification: Post Channel Assembly

Using a 1mL syringe and a syringe pump, 50mM of sodium tetraborate pH 8.5 was pumped through the channel at 2µL/minute for 1 hour to activate the amino group. A solution of 1mg/mL Sulfo-SMCC in 50

mM sodium tetraborate pH 8.5 was then pumped through the channel at 2µL/minute for 1 hour. Coupling buffer (50mM sodium phosphate, 50 mM NaCl and 10 mM EDTA, pH 7.2) was pumped through the channel at 2µL/ minute for 2 minutes to remove unreacted Sulfo-SMCC. The pump and the channels were then placed in a cold room and a 400µM protein solution of either CysA or CysALDV was then pumped at a rate of 2µL/minute through the channel overnight.

Cell Seeding

HUVECs and 3T3 cells were cultured separately according to standard procedures. A peristaltic pump was used to load the cells into the channel. Pumping continued until cells were coming out of the other end of the channel. Bubbles that formed in the channel were pushed out by using the rounded end of tweezers to gently pushing on the top of the PDMS channel. Excess cells in media were pipetted on the holes of the channel to prevent the channel from drying out, and the channels were placed in an incubator at 37˚C for 30 minutes. Images were taken of each channel at the middle of the channel and close to the end of the channel on each side. These images were taken with a 5x objective on a Zeiss Axio Observer Fluorescent microscope. To test whether the cells were attached to the channel, the channels were washed with PBS at 5µL/ minute for 10 minutes. Images were again taken with a 5x objective.

Results

B-PEG Purity

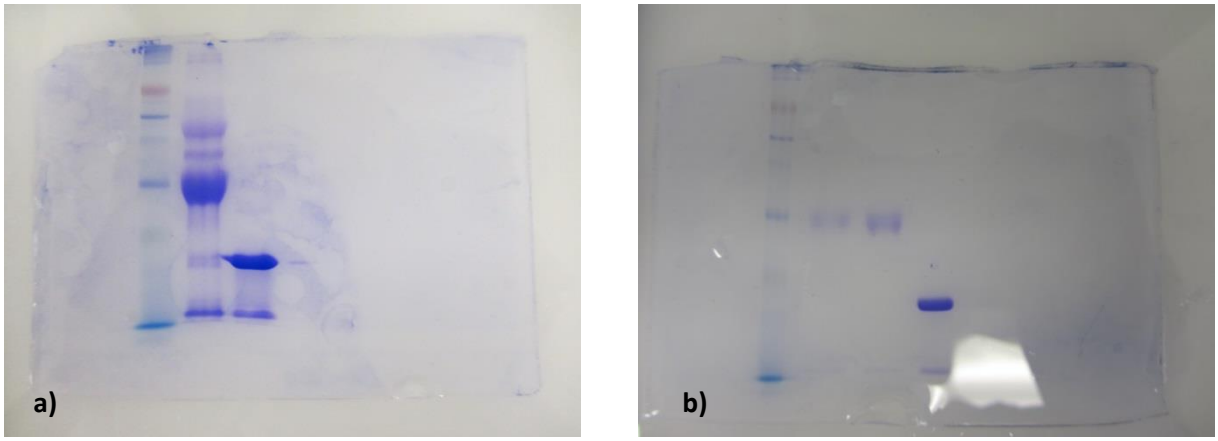

Figure 2: B-PEG SDS gels

a) Gel of the first B-PEG reaction. Left to right: ladder, B-PEG, BCys **b)** Gel of the second B-PEG reaction. Left to right: Ladder, B-PEG, concentrated B-PEG, BCys

Adhesion of Cells in Channels- Trial 1

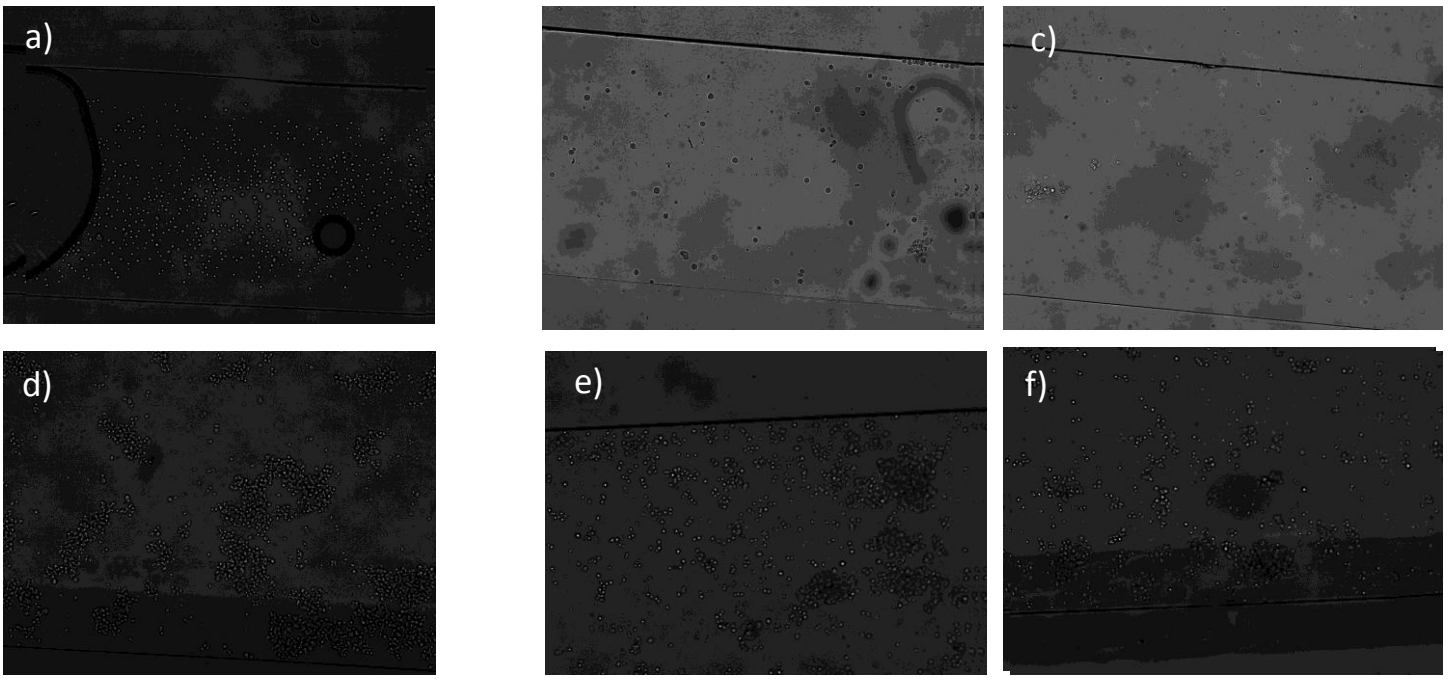

Figure3: Cys A Trial 1

a-c: 1mm wide 3T3 Channel **a)** Image taken between center and end of channel prior to the PBS wash. **b)** Image of channel between center and end, after PBS wash. **c)** Image of the center of channel, after the PBS wash.

d-f: 2mm wide HUVECs Channel **d)** Image taken of half of the width of the channel, between center and end of channel, before PBS wash. **e)** Image of half of the width of the channel, taken in the center of the channel, after PBS wash. **f)** Image of the other half of the center of the channel, after PBS wash. Note that the cells in e) and f) were moving.

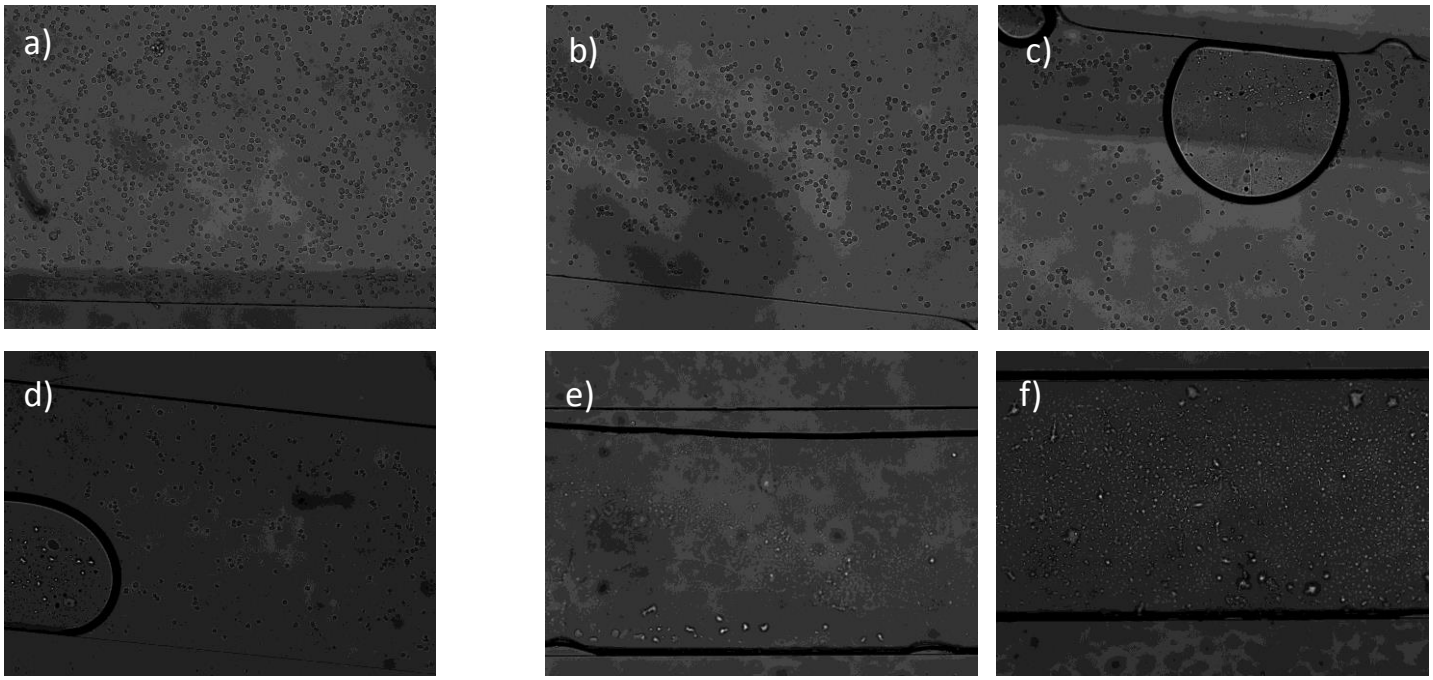

Figure 4: Cys ALDV Trial 1

a-c: 2mm wide 3T3 Channel **a)** Image taken of half of the width of the channel, between center and end of channel, before PBS wash. **b)** Image of half of the width of the channel, taken closer to one side of the channel. **c)** After PBS wash. Image of the other half of the center of the channel, after PBS wash.

d-f: 1mm wide HUVECs Channel **d)** Image taken between center and end of channel, before PBS wash. **e)** Image of channel

Adhesion of Cells in Channel- Trial 2

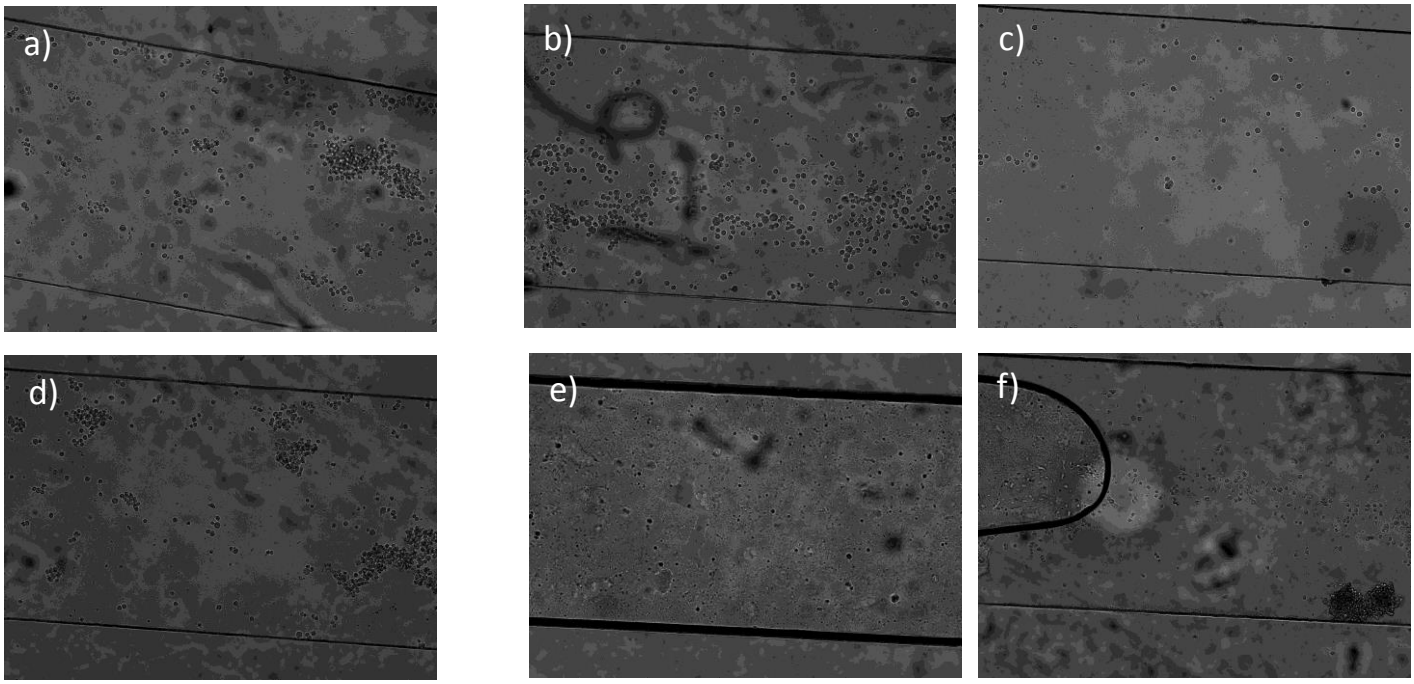

Figure 5: Cys A Trial 2

a-c: 1mm wide 3T3 Channel **a)** Image taken between center and end of channel, before PBS wash. **b)** Image of channel between center and end, after PBS wash. **c)** Image of the center of channel, after PBS wash.

d-f: 1mm wide HUVECs Channel **d)** Image taken of half of the width of the channel, between center and end of channel, before PBS wash. **e)** Image of half of the width of the channel, taken in the center of the channel. **f)** Image of the other half of the center of the channel, after PBS wash.

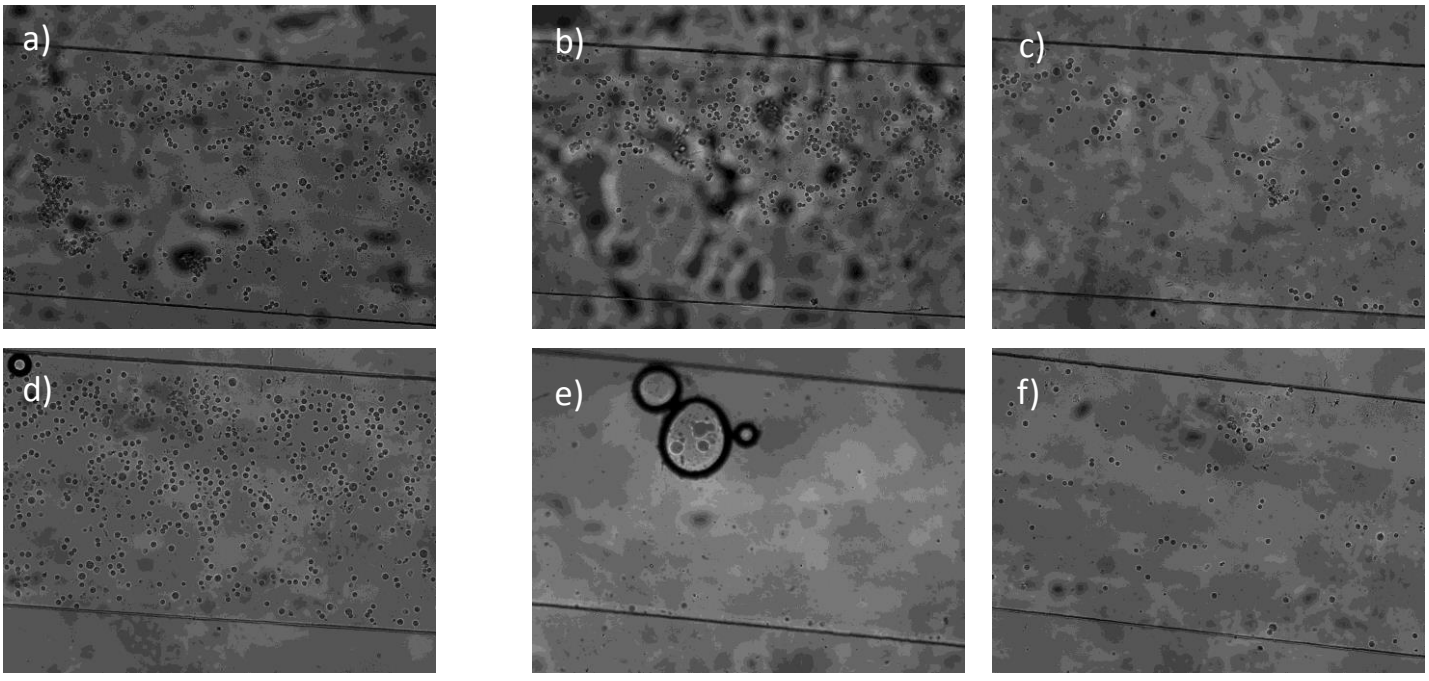

Figure 6: Cys ALDV Trial 2

a-c: 1mm wide 3T3 Channel **a)** Image taken at the middle of channel, before PBS wash. **b)** Image of channel between center and end, after PBS wash. **c)** Image of the center of channel after the PBS wash. Note that the cells in b) and c) were moving.

d-f: 1mm wide HUVECs Channel **d)** Image taken at the middle of the channel, before PBS wash. **e)** Image of the channel between the center and the end, after PBS wash. **f)** Image of the center of the channel, after PBS wash.

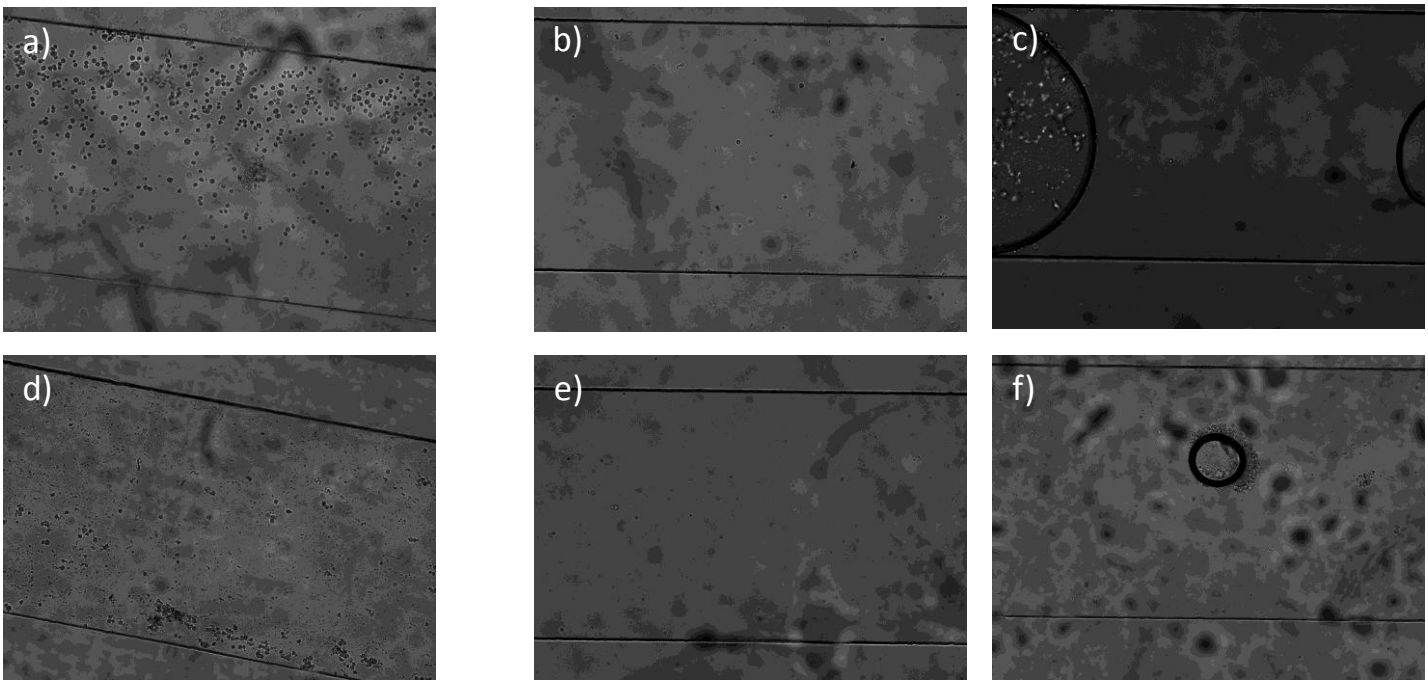

Figure 7: Cys ALDV Trial 2

a-c: 1mm wide HUVECs Channel **a)** Image taken at the middle of the channel, before PBS wash. **b)** Image of the center of channel, after PBS wash. **c)** Image of channel between center and end, after PBS wash.

d-f: 1mm wide HUVECs Channel **d)** Image taken between the center and the end of the channel, before PBS wash. **e)** Image of the channel between the center and the end, after PBS wash. **f)** Image of the other end of the channel, after PBS wash.

Discussion

Purity of B-PEG

The first attempt in react Bcys and PEG-Maleimide for the creation B-PEG was impure. This is shown in Figure 2a by the several bands above and below the large band. A band in the B-PEG column at the same height as the Bcys band indicated the Bcys had not been completely removed from B-PEG in the Thio-Sepharose column. Furthermore, the column had several prominent bands above the B-PEG band, indicating other impurities.

Prior to reacting Bcys and PEG-Maleimide for a second time, the Ni-NTA column was regenerated using NaOH and 8M urea with pH ranging from 4.5 to 8.0. This was done to eliminate the impurity bands above the original B-PEG band. The amount of binding buffer used to resuspend the B-PEG and Bcys after dialysis was reduced from around 20mL to 1mL, helping slow the flow rate from one drop every 13 seconds to one drop every 22 seconds. The slower flow rate gave the sample a longer contact time with the resin to remove more of the unwanted Bcys.

The purification of the second reaction between Bcys and PEG-Maleimide yielded a near pure product as seen in Figure 2b. A very faint band corresponding with the Bcys dimer band was seen on the concentrated B-PEG column, but was not visible on the original B-PEG column, indicating the presence is not prominent. A fainter band was also visible above the B-PEG band indicating other impurities, but the degree of faintness of the band indicated only a very weak presence and was considered to be negligible for the purposes of this experiment.

The original intent of the experiment was to verify a mechanism of adhesion for HUVECs, followed by a mechanism of release using B-PEG. Due to limited resources, the releasing of the HUVECs has been postponed.

Adhesion of Cells in Channel- First Trial

After the PBS wash, both the HUVECs and 3T3 cells in the CysA channels did not adhere a significant amount, as demonstrated in Figure 3. 3T3 cells were observed around the bubbles, however the cause of this is unknown. Furthermore, HUVECs were observed in the channel after the wash, however the cells moved when the channel was tipped, proving the cells were not adhered to the side of the channel, but rather were not completely washed out. Future trial should have a longer wash period or flow rate to ensure removal of the cells that were not adhered.

As seen in Figure 4, some HUVECs remained in the channels containing the CysALDV protein, however the 3T3 cells showed more cells in the channel after the PBS wash. This first seems to contradict the prediction that the HUVECs would adhere while the 3T3 cells would wash away, however a significant amount of bubbles were present in each channel, and these bubbles could have inhibited effectiveness of the PBS wash. This invalidated both the "adhesion" of the 3T3 and the HUVECs to the channel containing CysALDV in the first trial, however a correlation between a larger channel and more bubbles was found. This led to a future reduction of the channel size to 65μL deep for the next trial, along with only 1mm width used.

Adhesion of Cells- Second Trial

As seen in Figure 5, both channels modified with CysA had issues with cell aggregates. The 5μL/minute rate might not have generated enough force to move these aggregates even though they were not attached.

In the channels containing 3T3 and CysALDV, very few cells adhered. In images from Figure 6b-c, cells were present, however they were moving, thus indicating the PBS wash was not effective, but they were not adhered to the side.

Figure 7e-f shows that the channels containing the HUVECs in the CysALDV also failed to adhere many cells. Figure 7c shows that the initial cell seeding did not have a significant amount of cells, and thus did not have much opportunity for adhesion. Figure 7f also shows many cells within a bubble, indicating that is where the cells went.

Conclusion

In this study, proteins CysA and CysALDV were expressed, anticipating the HUVECs to adhere channel surfaces modified with CysALDV, while 3T3 adhered to neither. Due to complications with the flow rate and bubbles in the channels, the results were not what were expected, however they did not eliminate this mechanism as a possibility. Further work will be done to conclusively determine the efficiency of the CysALDV selectively adhering HUVECs. This study also synthesized B-PEG for the release of the

HUVECs once adhered to the CysALDV channel, however due to limited resources, this is an area that will also be worked on in the future.

Acknowledgements

The author thanks Wei Song for sharing his expertise on microfluidic channels and Dr. Wei Shen for her advice on microfluidic channels and experiment processes. The author also thanks Jeremiah Riesberg for culturing the HUVECs and 3T3 cells along with sharing his expertise on protein expression.

References

[1]Hye-Nam Son, H.; Ju-Ock Nam, J.; Kim, S.; Kim, I. Multiple FAS1 domains and the RGD motif of TGFBI act cooperatively to bind αvβ3 integrin, leading to anti-angiogenic and anti-tumor effects*Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* Oct2013; 2378–2388.

[2] Jellinghaus, S.; Poitz, D.; Ende, G.;Augstein, A.; Weinert, S.; Stütz, B.; Braun-Dullaeus, R.; Pasquale, E.; Strasser, R. Ephrin-A1/EphA4-mediated adhesion of monocytes to endothelial cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.* Oct. 2013; 2201–2211.

[3]Wang; Stuart Cooper. Adhesion of Endothelial Cells and Endothelial Progenitor Cells on Peptide-Linked Polymers in Shear Flow. *Tissue Engineering: Part A* 2013; 19, 1113-1121.

[4]Liu, B.; Liu Y.; Riesberg, J; Shen, W. Dynamic Presentation of Immobilized Ligands Regulated through Biomolecular Recognition. *Journal of American Chemical Society*. 2010; 132: 13630 13632.