Optimization of Protein Immobilization in Microfluidic Devices for Circulating Tumor Cell Capture

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The isolation of metastatic circulating tumor cells (CTCs) within the blood using microfluidic devices is a promising method for the detection of cancer. In this study, protein patterning of endothelial-leukocyte adhesion molecule-1 (E-selectin) and anti-epithelial-cell-adhesion-molecule (Anti-EpCAM) within microfluidic channels is utilized to improve the capture efficiency. To create this protein patterning the protein immobilization method must first be optimized to allow for maximum capture of CTCs and minimal increase in the flow rate of cells through the device due to added coating thickness. Proteins are immobilized in alternating regions using photo-initiated graft polymerization of polyacrylic acid (PAA) and a silanization reaction. Using interferometer measurements and fluorescent tagging, PAA height was minimized and protein immobilization was optimized.

Introduction

Current statistics indicate that cancer is the second leading cause of death for adults in the United States. As 90% of these deaths are caused by metastatic cancer, it is cruel for doctors to be able to detect metastatic cancer early in patients. Metastatic cancer is distinguished by circulating tumor cells (CTCs) or epithelial tumor cells circulating in the blood capable of creating secondary tumor sites. The ability to identify and filter these CTCs from the blood with high efficiency and purity would result in a much less invasive method of detecting and characterizing cancer cells when compared to the currently used biopsy method.

Due to the rarity of CTCs in the blood (1 per $10^9$ hematologic cells) a highly efficient method of CTC isolation from the blood is necessary. Recently, microfluidic devices functionalized with the antibody anti-epithelial-cell-adhesion-molecule (Anti-EpCAM) have been used to isolate CTCs. In the study presented here, Anti-EpCAM immobilization has been coupled with the immobilization of the protein endothelial-leukocyte adhesion molecule-1 (E-selectin) within the channels in the hopes of increasing efficiency of capture and furthering CTC isolation technology.

Anti-EpCAM is an effective molecule for CTC capture due to its affinity for the surface protein EpCAM present on CTCs. EpCAM is expressed in carcinomas of the lung, colorectal, breast, prostate, head and neck, and hepatic origin, and can therefore be used to capture a wide variety of cancer cells. In addition, it is not found on hematologic cells, meaning there should be no impurities. Despite the selectivity of Anti-EpCAM for CTCs, a high flow rate can decrease the efficiency of capture. This problem is combated by the use of E-selectin, which interacts with cell surface sialylated carbohydrates on both leukocytes and carcinoma cells. E-selectin binds cells with rapid bonding and breaking known as cell rolling. Cell rolling is ideal for pulling cells out of flow, and therefore can be utilized for slowing down CTCs to maximize cell contact with Anti-EpCAM, which will then securely bind the tumor cells. Although the E-selectin will also pull leukocytes out of flow, leukocytes lack the EpCAM surface molecule necessary to bind to Anti-EpCAM, and therefore will detach from the capture surface in regions functionalized with solely Anti-EpCAM.

Previous studies have shown that the use of a combination of E-selectin and Anti-EpCAM results in a three-fold improvement of separation and capture efficiency when compared to surfaces solely functionalized with Anti-EpCAM. In this study, E-selectin and Anti-EpCAM will be immobilized within the channel of a microfluidic device with specific patterning to optimize the capture efficiency of CTCs. By patterning the two proteins within the device with specific regions for Anti-EpCAM following Anti-EpCAM/E-selectin regions, white blood cells will detach from the E-selectin surface at the Anti-EpCAM region. This will inhibit build up of non-tumor cells on the capture surface. Ideally, this method of protein patterning will improve capture efficiency.

The microfluidic devices will be fabricated with soft lithography in polydimethylsiloxane (PDMS, ≥98%). In order to bind proteins within the channel, photo-initiated graft polymerization of polyacrylic acid (PAA) must be completed in areas of the channel designated by a photomask. PAA polymerization is desirable within the channel because it can be used to immobilize E-selectin and Anti-EpCAM through amide bonds. However, the PAA height can only be between 1-2 µm high to ensure the flow rate does not increase. If the PAA height were to exceed 2 µm, due to a constant pressure pumping method, the velocity would increase and the kinetics of CTC trapping would become unfavorable. An increased flow rate would lead to less cells being pulled out of the
flow by E-selectin, and would therefore decrease the capture efficiency of the experiment. Therefore, a major goal of this experiment is to manipulate variables in the photopolymerization reaction to minimize the height of the PAA coating.

In order to create the alternating regions of proteins, the channel areas without PAA polymerization will be backfilled by means of a silanization reaction utilizing the reagent 3-mercaptopropyl trimethoxysilane (MPTMS, 95%) and the crosslinker gamma-maleimidobutyryloxy succinimide (GMBS, ≥98%) to which proteins can then be attached. The attachment of Anti-EpCAM and E-selectin to the channel walls can be monitored using fluorescent labels. The efficiency of immobilization will be determined by measuring fluorescent intensity. These means of immobilization characterization will be used to optimize protein attachment for maximum capture efficiency. Once PAA height and protein immobilization have been optimized, flow experiments will be run to determine the speed of the cells through the channel and the efficiency of CTC capture.

**Materials and Methods**

**Fabrication of the Microfluidic Device**

The microfluidic devices used in this experiment were fabricated using soft lithography in PDMS. Molds were used to create PDMS channels, 10 parallel channel devices with a single inlet and outlet were used for flow experiments and single channel devices were used to test PAA height and protein immobilization. All chemicals used in this study were purchased from Sigma Aldrich and proteins were purchased from R and D Systems (Minneapolis, MN). Channels were 40 mm long, 660µm wide, and 90 µm tall. 1 ml of a 10:1 mixture of PDMS prepolymer and curing agent was degassed and spun onto a glass slide at 1000 RPM for 60 seconds and cured at 75°C for 30 minutes. To create the channels, 10 g of the 10:1 prepolymer and curing agent mixture was poured into a mold, degassed, and cured at 75°C for 90 minutes. The two PDMS layers were then plasma bonded to one another and heated at 65°C for 5 minutes.

**Photopolymerization**

The channels of the device were injected with 10% benzophenone (BP, ≥99%) in acetone continuously for 10 minutes. Nitrogen gas was then blown through the channels to displace the remaining BP. The devices were put in a vacuum for 15 minutes, then directly afterwards put in a nitrogen environment to displace the oxygen, which quenches the polymerization reaction. The channels were then injected with degassed (90 minutes) 20% acrylic acid (AA, ≥99%) monomer in water and exposed to UV light of 0.4 J/cm² intensity at the source and a rate of 2.2 mW/cm² at the target. Directly following Ultraviolet (UV) exposure the channels were rinsed with water then ethanol, and water was used to fill the channels while they were stored.

**TC Staining**

Toluidine Blue (TC, ≥98%) was diluted to 1% in water and adjusted to a pH of 10 using Sodium hydroxide (NaOH, ≥98%). The TC stain was then injected into the channels, which were then incubated at room temperature for 10 minutes. The channels were rinsed with water and the stained polymers were then visualized using bright field microscopy on an Olympus Premier Microscope and the PAA heights were measured with an interferometer.

**Measuring PAA Heights Using an Interferometer**

After photopolymerization was completed, the PAA heights in the channels were measured using an interferometer. To do this, the ceiling and floor components of previously plasma bonded PDMS slabs were separated from each other so PAA heights could be visualized with the device. The PAA height on the PDMS slab that was the top of the device will be referred to as the PAA ceiling, and the PAA height on the PDMS slab that was the bottom of the device will be referred to as the PAA floor. The device was zeroed in the closest surrounding areas in the channel without polymerization. In each channel, the regions 3, 8, 13 and 18 out of the 20 regions created were divided into four quadrants from which height measurements were taken.

**E-selectin and Anti-EpCAM Immobilization**

Following photopolymerization, the devices were vacuum dried for 30 minutes. Silanization was completed in a nitrogen atmosphere glove bag. 4% MPTMS in ethanol (99.5%) was injected into the channels and left to incubate 1 hour at room temperature. Afterward channels were flushed with ethanol. Proteins were then attached via amide bonds by rinsing the device with 1% Tween in Phosphate Buffered Saline (PBS, ≥98%) and allowing them to equilibrate for 5 minutes. A mix of 20 µg/mL E-selectin and 10 µg/mL Anti-EpCAM in PBS with 100X 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-Hydroxysuccinimide (NHS, ≥98.5%) was then injected into the channels and allowed to incubate for 2 hours at room temperature. Afterwards the channels were rinsed with 1% Tween in PBS and flushed with PBS. To immobilize proteins on GMBS, the devices were rinsed with ethanol and incubated in 0.002g GMBS dissolved in 100 µl DMSO in 1mL ethanol for 30 minutes at room temperature. The channels were then rinsed with...
ethanol and injected with 10 µg/mL Anti-EpCAM. This was allowed to incubate at room temperature for 2 hours, after which the devices were rinsed with PBS and treated with 1% Bovine serum albumin (BSA, ≥98%) for 30 minutes at room temperature. The devices were then rinsed with PBS a final time.

Characterization by Fluorescent Microscopy

Once the channel surfaces were functionalized with proteins, the channels were incubated with 1 µg/mL EpCAM in PBS for 30 minutes at room temperature. The devices were then rinsed with PBS and injected with a mixture of 10 µl Anti-EpCAM conjugated to Allophycocyanin (APC, ≥98%) and 10 µl Anti-human E-selectin-Fluorescein per 100 µl PBS. The channels were then incubated for 45 minutes at 2-8°C. Afterwards the channels were rinsed with PBS and imaged with Fluorescein isothiocyanate (FITC, ≥98%) and APC filters with an Olympus Premier inverted microscope and MetaMorph Software.

Cell Velocity Measurements

HL-60 human leukemia cells were centrifuged at 500 relative centrifugal force (RCF) for 5 minutes and resuspended in 2 mL of 4 µmol Calcein AM in PBS. The cells were then incubated at 37°C for 30 minutes. Afterwards, the cells were centrifuged at 500 RCF for 5 minutes and resuspended in PBS with cations. This step was repeated, then cells were mixed with 15 µl Trypan blue. The cells were allowed to incubate 5 minutes at room temperature, and then live white cells were counted using a hemocytometer on a light microscope at 20X. The concentration of cells was then adjusted to 10,000 cells/mL.

The stained cells were then drawn into a 3 ml syringe attached to 8G tubing with a 16 G needle. The syringe was attached to a syringe pump and the end of the tubing was attached to the inlet of the device. The Olympus microscope was then focused at 10X with the FITC filter and the cell solution was run through the device. 10-minute time lapses of 1 picture per second were taken of the devices at 8 µl of 10,000 HL-60 cells per mL per minute flow rate. The time lapse was taken, PBS without cations was pumped through the device for 10 minutes, and afterward the number of cells captured in the device was counted using florescent tagging. The number of cells entering the device was then manually counted from the time-lapse picture of the channel entrance, and the capture efficiency of the device could then be calculated by comparing the number of cells that entered the device and the number of cells that were captured.

Results

Cell Velocity Measurements

The data from the flow rate experiments compare the flow rates of HL-60 cells through channels with modified PAA surfaces and unmodified PDMS. The data shows the velocity of cells at different distances from the channel walls. The results indicate that the PAA modified surfaces at various flow rates have higher cell velocities than the PDMS surfaces at corresponding flow rates (Fig. 1). Therefore, to decrease flow rate through the device PAA height must be decreased.

PAA Height Optimization

Several factors have been identified that yield the optimum PAA height of 1-2 µm. The factors manipulated were bottom thickness, UV exposure energy, BP incubation time. It was found that the most influential factor in
FIG. 1: The velocities of HL-60 cells through devices modified with PAA and unmodified PDMS at varying flow rates. The data indicates that PAA surface modification leads to increased cell velocity.

FIG. 2: In preliminary tests the PAA heights in the microfluidic devices were much higher than the desired 2 \( \mu \text{m} \) at higher exposure energies, yet PAA growth did not occur at exposures lower than 0.5 Joules. These results indicated a change of procedure had to be made. PAA height growth was total UV exposure energy. Preliminary tests yielded heights of 4-6 \( \mu \text{m} \), with no growth when the exposure energy was below 0.5 J (Fig. 2). It was determined that these results were caused by oxygen interfering with the reaction, and subsequently the monomer was degassed. Reactions with the degassed monomer yield PAA heights below 2 \( \mu \text{m} \), and 0.4 J was determined to be the optimum UV exposure energy for the reaction after analysis with the interferometer and bright field microscopy (Fig. 3). The results also determined that the most consistent low PAA heights resulted from a 10 minute continuous injection period of the photo-initiator BP. Consequently, this method will be used for the PAA photopolymerization protocol.

FIG. 3: The most consistent PAA height of 2 \( \mu \text{m} \) occurred when the UV exposure energy was 0.4 Joules at the source after degassing the AA monomer was added to the procedure. This exposure energy will therefore be used for the rest of the experiment.

FIG. 4: The most consistently low PAA heights resulted from a 10 minute continuous injection period of the photo-initiator BP. Consequently, this method will be used for the PAA photopolymerization protocol.

E-selectin Immobilization

Once the PAA height results became consistent at 2 \( \mu \text{m} \), E-selectin immobilization was completed on the PAA surface. The initial results of fluorescent tagging showed that E-selectin immobilized well on the surface exposed to 0.4 J UV light based on average fluorescent intensity readings with the background noise subtracted (Fig. 5). Two procedures were tested, a 1-step procedure adding the E-selectin and EDC and NHS at the same time, and a 2-step procedure in which these steps were done separately. The 1-step procedure exhibited E-selectin immobilization with less noise when visualized with an FITC filter when samples when exposed to 0.4 J of UV light (Fig. 6).

Capture Efficiency

The cell capture studies ran MCF-7 cells through channels patterned with E-selectin/Anti-EpCAM and Anti-EpCAM. These studies tested the 1-step and 2-step E-selectin attachment procedures, and additionally the 1-step procedure was also tested with a buffer B solution (0.1 M MES (2-(N-morpholino)ethanesulfonic acid), pH
FIG. 5: The average fluorescent intensity measurements showed E-selectin immobilization in channels with PAA growth that had been exposed to 0.4 Joules of UV light over 180 seconds. The results suggest the 1-step method allows the most E-selectin immobilization on the PAA surface.

FIG. 6: The FITC imaging of channels exposed to 0.6 J UV light and immobilized with E-selectin. The top channel is done with the 1-step E-selectin immobilization procedure and clearly has less noise than the image on the bottom, which was immobilized with the 2-step procedure.

7.2, 0.5 M NaCl) in which the E-selectin is mixed with before it is added to the channel. These results indicate that the 1-Step E-selectin immobilization with buffer B results in the highest cell capture efficiency in devices patterned with E-selectin/Anti-EpCAM and Anti-EpCAM regions. However, the error is too large to be certain it is more efficient than the other methods. (Fig. 7)

Conclusion

Based on these results, the protocols for photopolymerization of PAA and E-selectin immobilization were optimized to levels that in theory will yield maximum efficiency for CTC capture in the PDMS devices. The results from the cells velocity experiment indicate that PAA height increases cell velocity as it travels through the device. Since increased cell velocity decreases capture efficiency, the optimum PAA heights within the channels are the lowest consistent levels possible. By altering several factors involved in the photopolymerization process, it was determined that by degassing the monomer, exposing the devices to 0.4 J of UV light over 180 seconds, and continuously pumping BP through the channels for 10 minutes yielded the most consistent results of around 2 μm PAA heights. Therefore, this treatment plan was incorporated into the procedure for making devices for CTC capture. Additionally, the results from the E-selectin immobilization experiments suggest that the 1-step method with buffer B yields the highest capture efficiency and protein immobilization. However, the error in these results is too high to be conclusive proof and therefore more trials of these experiments need to be run to determine the best E-selectin immobilization method.

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