PATTERNING OF ALTERNATING PROTEINS INSIDE A MICROFLUIDIC CHANNEL FOR ENHANCED TUMOR CELL ISOLATION
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ABSTRACT
Here we report on a technique to improve the capture rate of an affinity-based microfluidic CTC isolation device by patterning regions of alternating capture proteins. Of the two capture proteins, anti-epithelial cell adhesion molecule (aEpCAM) provides the specificity for CTC capture, while E-selectin endows robustness that increases CTC capture under shear. This patterning technique may also be useful in other biotechnology applications, as it can be used to spatially control the immobilization of any 2 proteins or protein mixtures inside a sealed microfluidic channel.

KEYWORDS: Circulating Tumor Cell, Microfluidics, Protein Immobilization, Patterning

INTRODUCTION
Due to the epithelial origin of most CTCs, aEpCAM is used for immunoaffinity separation. In existing microfluidic devices, capture with aEpCAM is limited by shear and therefore low flow rates are required for efficient capture [1]. E-selectin is an adhesion molecule expressed on endothelial cells, which has evolved specifically to bind cells in rapid blood flow. E-selectin exhibits catch bond behavior and rapid bond kinetics making it ideal for pulling CTCs out of flow. With the addition of E-selectin to a capture surface, CTCs can be isolated more efficiently than is possible with aEpCAM alone.

THEORY
While E-selectin binds to most CTCs, it also binds to leukocytes, which roll on E-selectin functionalized surfaces under flow. During the immune response, leukocytes experience initial tethering and rolling on E-selectin followed by firm adhesion to integrins. We mimic this natural process in a microfluidic device by combining E-selectin for initial CTC tethering with aEpCAM for firm adhesion. Previously we have shown that the addition of E-selectin can improve carcinoma cell capture efficiencies up to 3 fold over capture surfaces functionalized with only aEpCAM [2]. However, since E-selectin also binds leukocytes, it is necessary to pattern these capture proteins to reduce leukocyte build up. In this study the entire capture surface of the microfluidic device is functionalized with aEpCAM, while regions of E-selectin are interspersed with regions without E-selectin. As a result of this configuration, leukocytes can roll off the E-selectin regions and detach.

EXPERIMENTAL
The immobilization procedure for creating contiguous alternating regions of aEpCAM and aEpCAM + E-selectin mixture is outlined in Figure 1. Surface photopolymerization of acrylic acid was used to define regions on the polydimethylsiloxane (PDMS) channel surfaces. First benzophenone (BP) photoinitiator was absorbed into the PDMS channel surfaces by pumping 10% BP in acetone though the channel for 10 minutes. The device was flushed with nitrogen and dried under vacuum for 15 minutes to remove the acetone. The devices were transferred to a nitrogen filled glove bag for 15 minutes. A solution of 20% acrylic acid monomer in water was degassed for 1.5 hours to remove oxygen. The degassed monomer solution was injected into the channel immediately after the device was removed from the nitrogen environment. The bottom of the device was exposed through a photomask to 2.2 mW cm⁻², 375 nm UV radiation for 180 seconds (Figure 1B). The UV radiation activated the photoinitiator allowing polymerization to occur at the PDMS surface in the regions defined by the photomask. The device was flushed with water to remove the monomer and terminate the polymerization reaction and then flushed with ethanol to remove the BP. This polymerization reaction created a surface with alternating regions of PDMS and 1 to 2 µm thick polyacrylic acid (PAA) (Figures 1C and 2A). This resulted in regions on the channel walls presenting carboxyl groups to which aEpCAM or protein mixture can be attached. Silanization of the remaining exposed PDMS surfaces was carried out with a sulfhydryl-functionalized silanization reagent (4% v/v in ethanol 3-mercaptopropyl trimethoxysilane, MPTMS). After rinsing with ethanol and then PBS containing 0.5% Tween 20 (PBST), the PAA surface was functionalized with a soluble cross linker in PBS (0.8 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC and 0.8 mM N-Hydroxysuccinimide, NHS) for 30 minutes. Without rinsing, 10 µg/mL aEpCAM or a mixture of 10 µg/mL aEpCAM and 20 µg/mL E-selectin in PBS was injected into the channel and incubated for 2 hours. After the device was rinsed with PBST, an amine-to-sulfhydryl crosslinker (.01 mM in ethanol γ-maleimidobutyloxy succinimide, GMBS) was attached to the MPTMS for 30 minutes followed by rinsing with ethanol. Subsequently, 10 µg/mL aEpCAM or a mixture of 10 µg/mL aEpCAM and 20 µg/mL E-selectin in PBS was reacted with the GMBS for 2 hours, followed by rinsing with PBS. Finally, the channel was treated with 1% bovine serum albumin in PBS for 30 minutes to cover any remaining surfaces and block nonspecific interactions. Figure 2B shows fluorescently stained E-selectin and aEpCAM patterned on a channel surface using this method.

Devices were also fabricated with simple, non-patterned PAA or silane based protein immobilization chemistries. This fabrication consisted of protein immobilization onto PAA using EDC/NHS or immobilization onto the MPTMS silanization reagent using GMBS as described above including the BSA treatment.
Figure 1: Procedure for immobilizing alternating proteins on the interior surfaces of a sealed PDMS microfluidic channel. Both the NHS ester (orange) and carboxyl (green) presenting surfaces immobilize proteins through amide bond formation with protein amine groups. They are distinguished by the carboxyl presenting surface requiring a soluble cross linker, while the NHS ester presenting surface is itself a crosslinker.

Similar photopolymerization procedures have been used to pattern hydrophilic regions [3] or protein gradients [4] inside a sealed microfluidic channel. However, the new technique presented here combines photopolymerization with silanization backfilling to pattern alternating regions of proteins without the need for mask alignment.

Cell capture experiments were carried out with HL-60 and MCF-7 cells (used as white blood cell and CTC models respectively) suspended in phosphate buffered saline with cations (PBS). Cell were purchased from the American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. For easy visualization cells were stained with 4 μM Calcein AM in PBS at 37°C in the dark for 30 minutes before use. Cell suspensions at 10⁴ cells mL⁻¹ were hydrodynamically processed using a syringe pump and tubing positioned vertically to reduce the effects of cell settling. A flow rate of 8 μl min⁻¹ was used in all experiments for a shear rate of 15 s⁻¹ at the capture surface. For exact capture efficiency measurements, all incoming cells were imaged on an inverted microscope with a FITC filter as they entered the device, as described previously [5]. Incoming cells were automatically enumerated through image analysis. Devices consisted of 10 parallel channels, each with dimensions 90 μm x 660 μm x 40 mm. Channels were functionalized with αEPCAM only, E-selectin + αEPCAM mixture, or a pattern of alternating 1 mm regions of αEPCAM and protein mixture. In each device, proteins were immobilized using PAA based chemistry, silane based chemistry or both in the case of the patterned devices. In “pattern 1” devices the protein mixture was immobilized using PAA and αEPCAM was immobilized using the silane based chemistry. In “pattern 2” devices αEPCAM was immobilized using PAA and the protein mixture was immobilized using the silane based chemistry. Cell suspensions were injected into the device for 10 minutes followed by device rinsing with PBS without cations for 20 minutes. After stopping the flow, the number of captured cells were enumerated and divided by the number of cells which entered the device to get capture efficiency.

Figure 2: (A) 2μm thick surface patterned polyacrylamide in 90μm height channels. (B) Alternating regions of fluorescently stained αEPCAM (red) and E-selectin (green) immobilized on PDMS channel surfaces.
RESULTS AND DISCUSSION

Flow studies were carried out to test the efficacy of the patterned surfaces. Figure 3 shows the MCF-7 and HL-60 cell capture efficiencies of devices functionalized with aEpCAM, aEpCAM + E-selectin mixture, or patterned alternating regions of protein mixture and aEpCAM.

The patterned surfaces, which have half the surface functionalized with E-selectin, had on average 1.5 times the carcinoma cell capture efficiency of the aEpCAM only surfaces. This was only a 10% loss in carcinoma cell capture efficiency from the mixture functionalized surfaces, which are completely covered with E-selectin. The patterned surfaces had on average 40% the HL-60 cell capture efficiency of the mixture coated surfaces. This was a 7% increase in capture efficiency over the aEpCAM only functionalized surfaces.

CONCLUSION

Patterning alternating regions of aEpCAM and E-selectin + aEpCAM protein mixture provides a way to take advantage of the increased capture efficiency of this biomimetic protein combination while reducing leukocyte impurity build-up. These patterned surfaces can be integrated into complete CTC isolation systems for applications in research, cancer diagnosis and disease monitoring.

REFERENCES:

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