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Channel Surface Patterning of Alternating Biomimetic Protein Combinations for Enhanced Microfluidic Tumor Cell Isolation

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Abstract

Here we report a new method for multi-component protein patterning in a microchannel and also a technique for improving immunoaffinity-based circulating tumor cell (CTC) capture by patterning regions of alternating adhesive proteins using the new method. The first of two proteins, anti-epithelial cell adhesion molecule (anti-EpCAM), provides the specificity for CTC capture. The second, E-selectin, increases CTC capture under shear. Patterning regions with and without E-selectin allows captured leukocytes, which also bind E-selectin and are unwanted impurities in CTC isolation, to roll a short distance and detach from the capture surface. This reduces leukocyte capture by up to 82%. The patterning is combined with a leukocyte elution step in which a calcium chelating buffer effectively deactivates E-selectin so that leukocytes may be rinsed away 60% more efficiently than with a buffer containing calcium. The alternating patterning of this biomimetic protein combination, used in conjunction with the elution step, reduce capture of leukocytes while maintaining a high tumor capture efficiency that is up to 1.9 times higher than the tumor cell capture efficiency of a surface with only anti-EpCAM. The new patterning technique described here does not require mask alignment and can be used to spatially control the immobilization of any 2 proteins or protein mixtures inside a sealed microfluidic channel.

INTRODUCTION

CTCs are cells that have dissociated from a tumor site and entered the blood or lymph circulation. Some populations of these cells have the ability to extravasate and colonize distant tissues in a deadly process known as tumor metastasis. Analysis of CTCs can yield a wealth of information, making them highly valuable -both clinically and in basic research - in the effort to combat cancer and the metastatic process.¹ Methods for CTC isolation and characterization based on their “physical properties, expression of biomarkers, or functional characteristics” have been reviewed by Yu *et al.*² Antibody-based capture is the most common method of CTC isolation and anti-EpCAM is the most commonly employed antibody. This is due to anti-EpCAM’s variable but nearly universal expression in carcinoma cells and lack of expression in blood cells. In existing microfluidic devices, capture with anti-EpCAM is limited by shear and the low flow rates that are required for efficient capture.³ We propose to overcome these limitations with a second capture protein: E-selectin.

E-selectin is an adhesion molecule expressed on endothelial cells. This molecule has evolved specifically to bind cells in blood flow by forming catch bonds, with rapid binding kinetics, with its ligands on leukocytes and CTCs.^{4–6} The related P-selectin receptor has been indicated for lateral displacement-based cell separation and analysis under flow.^{7–8} Also, E-selectin has been combined with TNF-related apoptosis inducing ligand in an application to induce apoptosis of CTCs under flow conditions.^{9–10}

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Both CTCs and leukocytes 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 tumor cell tethering with anti-EpCAM for firm adhesion.

In this device, E-selectin pulls cells out of flow and increases the interaction between the cells and anti-EpCAM. Once target cells form initial bonds with E-selectin, the change in Gibbs free energy for anti-EpCAM binding to cell surface EpCAM is decreased (it becomes more negative). This is the result of enthalpic contributions from the decrease in cell velocity and entropic contributions from the cells' partial immobilization on E-selectin. The loss of rotational and translation entropy upon E-selectin binding prepay some of the entropy penalty for the immobilizing anti-EpCAM binding. This decrease in the change in free energy of binding leads to a higher percentage of binding events per contact with the binding surface. We demonstrate here and in previous work^{11, 12} that, with the addition of E-selectin to a capture surface, CTCs are captured more efficiently under flow than is possible with anti-EpCAM alone.

Importantly, E-selectin also binds leukocytes. Since blood comprises about 1% leukocytes and as few as 1 CTC per billion hematological cells, it is necessary to minimize leukocyte build up on the surface to prevent interference with CTC capture. This can be achieved by patterning the capture proteins on the surface. In this study the entire capture surface of the microfluidic device is functionalized with anti-EpCAM, while regions of E-selectin are interspersed between regions without E-selectin. As a result of this configuration, leukocytes roll to the edge of E-selectin regions, where binding destabilization and detachment occur due to a lack of specific interactions to maintain adherence (figure 1a).

After a sample is processed in the capture step, tumor cells are firmly bound to anti-EpCAM and a rinsing step can be executed to remove remaining leukocytes. During rinsing, calcium-dependent E-selectin can be deactivated by removing calcium with an elution buffer. Consequently, the specific interactions with E-selectin are blocked and leukocytes can be more effectively rinsed out of the device while anti-EpCAM binding to tumor cells is unaffected.

The in-situ dual protein patterning procedure described below takes advantage of the light permeability of polydimethylsiloxane (PDMS) to pattern alternating proteins inside a permanently bonded microfluidic device. Photopolymerized polyacrylic acid (PAA)¹¹ on channel surfaces is used both as a chemistry for protein attachment (similar to the use of polyacrylamide as described by Schneider *et al.*¹²) and as a protective layer for spatial control of protein immobilization using a second attachment chemistry, a silane backfill.

Several methods for multicomponent protein patterning have been developed recently¹³ to meet demands in fields such as biointerface science, biosensing, proteomics and smart materials. The different methods are based on photolithographic patterning^{14–21}, photochemical immobilization^{22–27}, or microcontact printing^{7, 28}. Each of these methods uses either identical or different attachment chemistries to immobilize different proteins. In this new technique, both proteins are immobilized by amide bond formation between surface carboxyl groups and protein amine groups. The two different attachment chemistries are distinguished only by their requirements for soluble crosslinkers. Therefore this method can be used to immobilize any two proteins or protein mixtures.

Techniques involving microcontact printing or spin coating are often used in conjugation with pressure sealed flow chambers to avoid complications arising from plasma bonding (i.e. the deactivation or ablating away of surface treatments). While this allows a wider range of surface modifications, it limits the versatility of the surfaces in terms of device

functionality. Because the entire process is carried out in a sealed channel, the double patterning technique described here can be incorporated into almost any PDMS device, regardless of its complexity or the processing required for assembling it.

Photolithographic patterning techniques involve the selective removal or deposition of photoresist to expose or cover reactive chemistries or adsorption surfaces. Proteins can then be immobilized on the exposed surface, while the photoresist acts as a blocking reagent to prevent immobilization on the rest of the surface. Often the photoresist is then removed to backfill the remaining surface. With the technique described here, PAA is not only a blocking reagent for the silane backfill, but also the base for the other immobilization chemistry. Therefore the procedure is simplified because there is no photoresist removal step.

In the technique described here, the patterning is carried out after the channel is sealed, but before the proteins are immobilized. This avoids complications such as surface-modification effects on device bonding, bonding effects on surface modifications and biomolecule damage from patterning reagents or radiation. Also, because this patterning technique does not require mask alignment, the amount of resources needed to duplicate it is reduced. An additional benefit of this technique is that the reagents are inexpensive, readily available, and commonly used in other CTC isolation devices.^{3, 29}

This novel patterning process allows the spatially controlled immobilization of 2 proteins inside a permanently bonded PDMS channel. This process was developed specifically to create microfluidic capture surfaces with contiguous alternating regions of anti-EpCAM protein and anti-EpCAM plus E-selectin protein mixture (aE-ES) that allow optimum tumor cell sequestration with minimum leukocyte capture.

EXPERIMENTAL SECTION

Device Fabrication

Devices were fabricated in PDMS (10:1 elastomer to curing agent ratio) by standard soft lithography techniques. Glass slides were spin coated with PDMS at 1000 rpm for 1 minute. Devices consisted of 10 parallel channels (each with dimensions 90 μm $\times$ 660 μm $\times$ 40 mm) with a single inlet and outlet. The devices were plasma bonded to the coated slides with a hand-held corona discharge wand (Model BD-20, Electrotech Products Inc., Chicago, IL) immediately before micropatterning.

Micropatterning Alternating Proteins

The immobilization procedure for creating contiguous regions of alternating anti-EpCAM and aE-ES is outlined in figure 2. Similar photopolymerization procedures have been used to pattern hydrophilic regions¹¹ or protein gradients¹² inside a sealed microfluidic channel. However, this new technique combines photopolymerization with silanization backfilling to pattern alternating regions of proteins without the need for mask alignment. Proteins were purchased from R&D Systems (Minneapolis, MN), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Surface photopolymerization of acrylic acid was used to define regions on the PDMS channel surfaces in a similar manner as previously described¹². First benzophenone (BP, 99%) photo initiator was absorbed into the PDMS channel surfaces by injecting 10% BP in acetone through the channel for 10 minutes (swelling due to acetone allows BP to enter the PDMS) (figure 2a). The device was flushed with nitrogen and dried under vacuum for 15 minutes to remove the residual acetone and oxygen, as oxygen quenches the polymerization reaction. The devices were transferred to a nitrogen environment for 15 minutes. A solution

of 20% acrylic acid monomer (99.0%) in water was degassed for 1.5 hours to remove dissolved oxygen. Degassed monomer solution was injected into the channels under the nitrogen environment to reduce oxygen diffusion into the PDMS device (figure 2a). The bottom of the device was promptly exposed to 0.5 mW cm^{-1} , 375 nm ultraviolet (UV) radiation for 210 s through a photomask (figure 2a). The UV 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 solution and then flushed with ethanol to remove the BP.

This polymerization reaction was used to create channel surfaces with 1 mm, 600 μm , and 300 μm wide alternating regions of PDMS and PAA as shown in Figs. 1b and 2b. An asymmetric pattern with 1 mm PAA widths and 300 μm PDMS widths was also created. PAA film heights were .5 to 2 μm thick as determined using a custom built interferometer.³⁰ The PAA regions created by this photopatterning presented carboxyl groups for use in the attachment of the aE-ES (figure 2c) and also blocked those regions from PDMS modification by silanization (Fig 2b).

After placing the devices in a vacuum chamber for 30 minutes to remove oxygen and traces of water, silanization of the remaining exposed PDMS surfaces was carried out in a nitrogen filled glove bag with the sulfhydryl-functionalized silanization reagent 3-mercaptopropyl trimethoxysilane (MPTMS (95%), 4% v/v in absolute ethanol) (Fig 2b).

Next the PAA surfaces were functionalized by incubation for 2 hours in a mixture of anti-EpCAM (10 $\mu\text{g/mL}$) and E-selectin (3.9 $\mu\text{g/mL}$) in phosphate buffered saline without cations (PBS^-) with soluble crosslinker (0.8 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC and 0.8 mM N-Hydroxysuccinimide, NHS, 98.5%) (Fig 2c). Subsequently 0.01 mM of the amine-to-sulfhydryl crosslinker γ -maleimidobutyryloxy succinimide in ethanol (GMBS, 98.0%) was attached to the MPTMS (Fig 2d). 10 $\mu\text{g/mL}$ anti-EpCAM in PBS^- was reacted with the GMBS for 2 hours (figure 2e). Finally, the channel was treated with 1% bovine serum albumin to cover any remaining surfaces and block nonspecific interactions between cells and the capture surface. Figure 1c shows fluorescently stained E-selectin and anti-EpCAM patterned on a channel surface using this method.

Protein Immobilization on Non-patterned Surfaces

Non-patterned devices were also fabricated using silane or PAA based immobilization methods. Silane devices were made using MPTMS silanization reagent and GMBS, as described above, to immobilize anti-EpCAM only (10 $\mu\text{g/mL}$), aE-ES protein mixture (10 $\mu\text{g/mL}$ each) or (as a negative control) 10,000 MW methyl polyethylene glycol amine (PEG, 50 $\mu\text{g/mL}$). Non-patterned PAA devices were made by photopolymerization of acrylic acid followed by immobilization of proteins using EDC/NHS soluble cross linker, as described above. Non-patterned PAA devices included anti-EpCAM only (10 $\mu\text{g/mL}$), aE-ES (10 $\mu\text{g/mL}$ anti-EpCAM and 3.9 $\mu\text{g/mL}$ E-selectin) and PEG.

Surface Characterization

The protein functionalized surfaces were characterized by immunostaining as described in the supporting information.

Flow experiments

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^+). Cells were purchased from the American Type Culture Collection (ATCC) and

cultured according to ATCC recommendations. For easy visualization, cells were stained with Calcein AM^{10, 31} in PBS⁺ at 37°C in the dark for 30 minutes before use. Homogeneous cell suspensions were stained with 4 μ M Calcein AM. For the cell mixtures, HL-60 cells and MCF-7 cells were stained with 1 μ M, and 12 μ M Calcein AM, respectively. Cell suspensions of 10⁴ HL-60 or MCF-7 cells mL⁻¹ in PBS⁺ were used as homogeneous cell suspensions. Cell mixture suspensions contained 10⁶ HL-60 and 10³ MCF-7 cells mL⁻¹. Cell suspensions were hydrodynamically processed using a syringe pump and tubing that were positioned vertically to reduce the effects of cell settling.³² A flow rate of 8 μ L min⁻¹ (shear rate of 15 s⁻¹ at the capture surface) was used for all experiments. 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 previously³². The two cell types in the cell mixture suspension were distinguished by size and fluorescent intensity (figure S-3). Incoming cells were automatically enumerated through image analysis. For visualization of cell capture, a 100 to 1 ratio of HL-60 to MCF-7 cells (10⁶ and 10⁴ cells per mL respectively) were processed in the device in the same manner as the cell capture experiments.

Channels were functionalized with: silane or PAA immobilized anti-EpCAM, silane or PAA immobilized aE-ES, a pattern of alternating anti-EpCAM and aE-ES with 1 mm, 600 μ m, 300 μ m or asymmetric spacing, or PEG as a negative control. For capture efficiency experiments, cell suspensions were injected into the device for 10 minutes followed by device rinsing for 10 minutes. A rinse buffer, containing the calcium specific chelating agent ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, 5 mM, 97.0%), and 3 mM MgCl₂ in PBS⁻ at 295 mOsm total, was used to remove leukocytes, without displacing captured tumor cells. After rinsing, the number of captured cells was enumerated and divided by the number of cells which had entered the device to calculate the capture efficiency. Rinsing studies were carried out in a similar fashion except cells captured on protein mixture functionalized surfaces were enumerated before and after rinsing with 295 mOsm PBS⁺, PBS⁻, PBS⁻ with 5 mM EGTA, or PBS⁻ with 5 mM EGTA plus 3mM Mg²⁺.

RESULTS AND DISCUSSION

Here we demonstrate a technique for increasing anti-EpCAM based tumor cell capture using E-selectin, and methods for releasing the additional leukocytes captured by the E-selectin.

One method for increasing leukocyte release is the complete removal of Ca²⁺ to deactivate the capture surface immobilized E-selectin during device rinsing. This can be done with PBS⁻, or with PBS⁻ plus EGTA with or without Mg²⁺. The MCF-7 and HL-60 cell retention rates after rinsing with the 3 different leukocyte elution buffers (plus PBS⁺ as a control) are presented in figure 3. The cell retention rate was calculated by taking the number of captured cells remaining after rinsing and dividing by the original number of captured cells counted before rinsing. There were no significant differences ($p > 0.05$) in carcinoma cell retention on silane aE-ES functionalized surfaces after rinsing with any of the 4 buffers. These surfaces had 92–95% retention of the MCF-7 carcinoma cells after the 10 minute rinsing period. This is expected, because the carcinoma cells are firmly adherent to anti-EpCAM. Conversely, these same surfaces had a high, 56%, leukocyte retention rate with the PBS⁺ rinse, which dropped significantly to 31%, 19% and 13% for the PBS⁻, EGTA, and EGTA plus Mg²⁺ buffers, respectively ($p < 0.001$). The increased rinsing efficiency with the EGTA solution is due to Ca²⁺ removal from the glycosylated ligand binding site on the E-selectin (figure S-2), which prevents E-selectin binding to leukocyte ligands. The effect of the Mg²⁺ is presumably the displacement of the Ca²⁺ ion from its binding site in E-selectin, for more complete Ca²⁺ removal.³³ A visualization of captured cells before and after rinsing

with elution buffer is presented in figures 1d and 1e. Although the elution buffer is 60% more effective at removing captured leukocytes during the rinsing step, it only rinses away 87% of the leukocytes captured during sample processing step. For this reason, another method is employed to reduce the number of leukocytes captured during the sample processing step.

The second method for reducing leukocyte capture involves patterning alternating regions with and without E-selectin. On this surface, captured leukocytes may roll a short distance and detach. Flow studies were carried out to test the efficacy of the patterned surfaces over monolithic surfaces. Devices were functionalized with: silane or PAA immobilized anti-EpCAM, silane or PAA immobilized aE-ES, a pattern of alternating silane anti-EpCAM and PAA aE-ES with 1 mm, 600 μm , 300 μm or asymmetric spacing, and silane or PAA immobilized PEG (negative control). These devices were tested with cell suspensions that were either homogeneous, with 10^4 cells mL^{-1} , or cell mixtures with 10^6 HL-60 and 10^3 MCF-7 cells mL^{-1} . In the cell mixture suspensions, the two cell types were stained with two different concentrations of Calcein AM. This allowed them to be distinguished, so both the MCF-7 and the HL-60 cell capture efficiencies could be determined for each trial (figure S-3). All devices were rinsed with the EGTA/ Mg^{2+} solution for 10 minutes after cell suspensions were processed through the devices.

Table 1 shows the results of the flow studies. It is organized, first, by the basis of protein immobilization (silane, PAA, or the pattern of alternating silane and PAA), and then by the proteins immobilized by this method (anti-EpCAM or aE-ES) or by the spacing of the pattern sections. The capture efficiencies are reported as the mean $\pm$ the standard error. The statistical analysis of the flow study results is summarized in table S-3.

Carcinoma Cell Capture

The goal of microfluidic immunoaffinity based CTC isolation is to separate CTCs from other blood components through specific binding to a capture surface. Due to the rarity of CTCs in blood, it is important to achieve a high CTC capture efficiency to limit the amount of patient blood required. Here we compared the capture efficiencies of the different capture surfaces listed above using MCF-7 cells as a CTC model.

As expected based on our previous results³⁴ and the known ability of E-selectin to pull cells out of flow, there was a significant increase in MCF-7 cell capture between the anti-EpCAM and aE-ES functionalized surfaces (table S-3 c and d). Adding E-selectin to the capture surface increased MCF-7 capture 1.6 fold for the silane surface and 1.7 fold for the PAA surface.

Alternately, there was not a significant difference in MCF-7 capture efficiencies between the aE-ES functionalized capture surfaces and any of the pattern functionalized surfaces, including the pattern surface tested with the cell mixture (table S-3 f). This demonstrates that the patterned capture surfaces were able to increase anti-EpCAM based capture as effectively as the aE-ES surface, (100% E-selectin coverage) despite having only 50% or 77% of their surface areas functionalized with E-selectin. This indicates a strong effect of E-selectin and possibly a maximum capture rate due to transport limitations in this flat-walled device. Stott *et al.* reported the use of herringbone mixer structures to improve transport and CTC capture from whole blood in an anti-EpCAM based isolation device.²⁹ If the patterned surfaces described here are incorporated into a complete CTC isolation system, mixing structures should be included to overcome transport limitations to allow maximum CTC capture efficiency. Because this surface modification technique is based on photopatterning (as opposed to contact based patterning), the technology is expected to translate well to

corrugated surfaces (such as mixer features), as other photopatterning technologies have.^{35–36}

The MCF-7 cell capture efficiencies increased up to 1.9 fold between the anti-EpCAM and patterned surfaces (table S-3 e and g). There was a similar result in the cell mixture flow studies. The 300 μm patterned surface had a significant 1.5 fold increase in MCF-7 cell capture efficiency over the anti-EpCAM surfaces (table S-3 h and i). There was a small decrease in anti-EpCAM based MCF-7 capture efficiency between the homogeneous cell suspensions and the cell mixture suspensions, indicating that a physiological concentration of HL-60 cells may interfere with MCF-7 capture. However, the difference was not statistically significant (table S-3 a), which supports the use of this technique with real biological samples.

The pattern geometry did not appear to have an effect on MCF-7 capture as there was not a significant difference in MCF-7 capture between any of the patterned surfaces (table S-3 f).

Leukocyte Capture

Leukocytes are the most common hematologic cell impurity captured in CTC isolation. This is because they are the most similar to CTCs in terms of the physical properties, expression of biomarkers, and functional characteristics that are the basis of CTC separations.² Therefore it is important to study the effects of different surface functionalizations on leukocyte capture. We have done so here using HL-60 cells as a leukocyte model.

Fluorescent characterization studies showed that a 3.9 $\mu\text{g mL}^{-1}$ E-selectin treatment of the PAA surface results in approximately the same surface density of E-selectin as a 10 $\mu\text{g mL}^{-1}$ E-selectin treatment of the silane surface. This is further supported by the flow studies, where the aE-ES PAA and aE-ES silane surfaces did not have statistically different HL-60 cell capture efficiencies (table S-3 o).

An important feature of E-selectin is its affinity for ligands on leukocytes. The effect of this affinity can be seen in the increase in HL-60 cell capture efficiency that occurs between the anti-EpCAM and aE-ES surfaces. The addition of E-selectin to the capture surface resulted in a 4.3 and 4.8 fold increase in HL-60 cell capture efficiency for the silane and PAA surfaces, respectively (table S-3 k and l). This unwanted increase in HL-60 cell capture can be negated by patterning the two capture proteins on the capture surface.

The pattern surfaces were effective at reducing HL-60 cell capture. This is demonstrated by a comparison between the HL-60 cell capture efficiencies of the pattern surfaces with the capture efficiencies of the negative controls (table S-3 j). Negative controls include HL-60 cell capture on anti-EpCAM and PEG surfaces, and MCF-7 cell capture on PEG surfaces. There was no significant difference between HL-60 cell capture on the pattern surfaces and the negative control surfaces (table S-3 j). This means that the HL-60 cell capture on patterned surfaces was not higher than the HL-60 cell capture caused by non-specific electrostatic interactions on the negative control surfaces and it supports the hypothesis that the combination of protein patterning and elution buffer effectively negate the specific interactions between leukocytes and capture surface E-selectin. Zheng *et al.*³⁷ demonstrated a technique applying variable shear in the rinsing step to preferentially remove captured non-target cells. This technique could be used to augment the methods of leukocyte removal discussed in this paper to further reduce non-specific capture.

Different pattern section widths were tested to study the effect of pattern geometry on cell capture. All patterned surfaces had significantly less HL-60 capture than the aE-ES surfaces

(table S-3 m and n). The most effective was the 300 μm pattern, which had 5.6 fold lower HL-60 cell capture than the aE-ES surface.

Unlike MCF-7 cell capture, HL-60 cell capture was variable for the different pattern geometries. As expected the asymmetric pattern, which has about 77% of its surface functionalized with E-selectin, had the highest HL-60 cell capture efficiency of all the patterned surfaces. The symmetric patterns (with 50% surface E-selectin coverage) had lower HL-60 cell capture efficiencies, which decreased with decreasing pattern width. Figure 4 illustrates the effects of E-selectin coverage and pattern dimensions on HL-60 cell capture. In the plot, capture efficiencies are normalized to the capture efficiency of the PAA aE-ES surface (100% E-selectin coverage). The plot shows a decrease in HL-60 cell capture with percent E-selectin coverage. It also shows a decrease in HL-60 cell capture with decreasing pattern width (independent of percent E-selectin coverage).

Although the differences in HL-60 cell capture for the different width symmetric patterns were not significantly significant in this study (table S-3 j), the correlation between pattern width and HL-60 cell capture indicates that the density of surface boundaries may be a factor in leukocyte release efficiency. Surfaces with higher boundary densities may have improved leukocyte removal for two reasons. A shorter rolling distance between the site of HL-60 cell capture and the adjoining anti-EpCAM region could reduce the probability that the cell will reach a localized region of higher E-selectin density and become arrested, before it can be released at the anti-EpCAM boundary. Additionally, increasing the number of boundaries increases the space where leukocyte binding is being destabilized and detachment is occurring, leading to more total leukocyte detachment. The key features of this work are the increase in anti-EpCAM based tumor cell capture efficiency under flow conditions and the minimization of leukocyte capture through protein patterning and Ca^{2+} removal. Calcium free buffer has been suggested for rinsing in E-selectin functionalized devices.¹⁰ Here we take that idea further with the molecular displacement and specific chelating of Ca^{2+} with Mg^{2+} and EGTA, respectively. Previously, Mg^{2+} /EGTA buffers were shown to abolish ligand binding to L-selectin but not P-selectin in affinity columns.³³ Now we have shown that this elution buffer is effective for disrupting leukocyte E-selectin binding in a microfluidic device.

E-selectin has been indicated for in-vitro cell binding under flow.⁹ Our group has expanded this idea by combining E-selectin with a capture molecule that provides immuno-specificity for CTC capture³⁴ and furthermore described surface patterning and elution methods for utilizing this protein combination while reducing leukocyte build-up on the capture surface.

Anti-EpCAM was co-immobilized with E-selectin in this study as an example of an immunospecific adhesion molecule. Other such molecules could also be co-immobilized with E-selectin for efficient capture of tumor cells with low EpCAM expression. It should be noted, however, that some CTC specific capture molecules (such as cadherins) are calcium-dependent and the leukocyte elution step is not compatible with surfaces functionalized with those molecules. Alternately, the elution buffer from this study could be used to recover CTCs captured with those molecules.

Increasing tumor cell capture under flow conditions is an important step towards reducing the shear dependency of immunospecific capture and increasing the throughput of CTC isolation, independently of parallelization. Parallelization will still be required to achieve a sufficient throughput for processing large quantities of blood. However, increasing throughput independent of parallelization will reduce the amount of parallelization required and reduce total costs of device production.

CONCLUSIONS

Here we reported a new multi-protein patterning technique that creates a surface pattern after a channel has been sealed, but before proteins are immobilized on the substrate, thereby avoiding many common complications associated with the immobilization of sensitive biomolecules. Patterning alternating regions of anti-EpCAM and biomimetic protein mixture provides a way to leverage the increased tumor cell capture efficiency of the protein combination while reducing leukocyte impurity buildup. Also, a leukocyte elution buffer consisting of Ca^{2+} chelating EGTA and Ca^{2+} displacing Mg^{2+} effectively deactivates E-selectin to increase the efficiency leukocyte release during device rinsing. After further validation with physiological samples, these patterned surfaces may be integrated into CTC isolation systems for applications in research, cancer diagnosis and disease monitoring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Paterlini-Brechot P, Benali NL. *Cancer Lett.* 2007; 253:180–204. [PubMed: 17314005]
2. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. *J Cell Biol.* 2011; 192:373–82. [PubMed: 21300848]
3. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA, Toner M. *Nature.* 2007; 450:1235–9. [PubMed: 18097410]
4. Tedder TF, Steeber DA, Chen A, Engel P. *FASEB Journal.* 1995; 9:866–73. [PubMed: 7542213]
5. Barthel SR, Gavino JD, Descheny L, Dimitroff CJ. *Expert Opin Ther Targets.* 2007; 11:1473–91. [PubMed: 18028011]
6. Long M, Zhao H, Huang KS, Zhu C. *Ann Biomed Eng.* 2001; 29:935–46. [PubMed: 11791676]
7. Lee CH, Bose S, Van Vliet KJ, Karp JM, Karnik R. *Langmuir.* 2011; 27:240–9. [PubMed: 21141947]
8. Hong S, Lee D, Zhang H, Zhang JQ, Resvick JN, Khademhosseini A, King MR, Langer R, Karp JM. *Langmuir.* 2007; 23:12261–8. [PubMed: 17949112]
9. Rana K, Liesveld JL, King MR. *Biotechnol Bioeng.* 2009; 102:1692–702. [PubMed: 19073014]
10. King MR, Western LT, Rana K, Liesveld JL. *Journal of Bionic Engineering.* 2009; 6:311–317.
11. Fiddes LK, Chan HK, Lau B, Kumacheva E, Wheeler AR. *Biomaterials.* 2010; 31:315–20. [PubMed: 19800682]
12. Schneider MH, Willaime H, Tran Y, Rezgui F, Tabeling P. *Anal Chem.* 2010
13. Ganesan R, Lee HJ, Kim JB. *Langmuir.* 2009; 25:8888–93. [PubMed: 19719213]
14. Jackson BL, Groves JT. *Langmuir.* 2006; 23:2052–2057. [PubMed: 17279694]
15. Katz JS, Doh J, Irvine DJ. *Langmuir.* 2006; 22:353–9. [PubMed: 16378444]
16. Kwon KW, Choi JC, Suh KY, Doh J. *Langmuir.* 2011; 27:3238–43. [PubMed: 21348500]
17. Waichman S, Bhagawati M, Podoplelova Y, Reichel A, Brunk A, Paterok D, Piehler J. *Analytical Chemistry.* 2010; 82:1478–1485. [PubMed: 20092261]
18. Li N, Ho CM. *Lab on a Chip.* 2008; 8:2105–2112. [PubMed: 19023473]
19. Chen S, Smith LM. *Langmuir.* 2009; 25:12275–12282. [PubMed: 19821627]
20. Reynolds NP, Tucker JD, Davison PA, Timney JA, Hunter CN, Leggett GJ. *Journal of the American Chemical Society.* 2009; 131:896–897. [PubMed: 19128000]

21. Doh J, Irvine DJ. *Journal of the American Chemical Society*. 2004; 126:9170–9171. [PubMed: 15281792]
22. Blawas AS, Oliver TF, Pirrung MC, Reichert WM. *Langmuir*. 1998; 14:4243–4250.
23. Holden MA, Jung SY, Cremer PS. *Analytical Chemistry*. 2004; 76:1838–1843. [PubMed: 15053641]
24. Belisle JM, Kunik D, Costantino S. *Lab on a Chip*. 2009; 9:3580–3585. [PubMed: 20024039]
25. Dillmore WS, Yousaf MN, Mrksich M. *Langmuir*. 2004; 20:7223–7231. [PubMed: 15301509]
26. Chan EWL, Yousaf MN. *Molecular BioSystems*. 2008; 4:746–753. [PubMed: 18563249]
27. Holden MA, Cremer PS. *Journal of the American Chemical Society*. 2003; 125:8074–8075. [PubMed: 12837056]
28. Bernard A, Renault JP, Michel B, Bosshard HR, Delamarche E. *Advanced Materials*. 2000; 12:1067–1070.
29. Stott SL, Hsu CH, Tsukrov DI, Yu M, Miyamoto DT, Waltman BA, Rothenberg SM, Shah AM, Smas ME, Korir GK, Floyd FP Jr, Gilman AJ, Lord JB, Winokur D, Springer S, Irimia D, Nagrath S, Sequist LV, Lee RJ, Isselbacher KJ, Maheswaran S, Haber DA, Toner M. *Proc Natl Acad Sci U S A*. 2010; 107:18392–7. [PubMed: 20930119]
30. Yen GS, Fujimoto BS, Schneider T, Huynh DT, Jeffries GD, Chiu DT. *Lab Chip*. 2011; 11:974–7. [PubMed: 21229183]
31. Abbitt KB, Rainger GE, Nash GB. *J Immunol Methods*. 2000; 239:109–19. [PubMed: 10821952]
32. Launier CA, Czaplewski GJ, Myung JH, Hong S, Eddington DT. *Biomed Microdevices*. 2011; 13:549–57. [PubMed: 21409456]
33. Koenig A, Norgard-Sumnicht K, Linhardt R, Varki A. *J Clin Invest*. 1998; 101:877–89. [PubMed: 9466983]
34. Myung JH, Launier CA, Eddington DT, Hong S. *Langmuir*. 2010; 26:8589–8596. [PubMed: 20155985]
35. Kim M, Choi JC, Jung HR, Katz JS, Kim MG, Doh J. *Langmuir*. 2010; 26:12112–8. [PubMed: 20565061]
36. Martin TA, Herman CT, Limpoco FT, Michael MC, Potts GK, Bailey RC. *ACS Appl Mater Interfaces*. 2011; 3:3762–71. [PubMed: 21793535]
37. Zheng X, Cheung LS, Schroeder JA, Jiang L, Zohar Y. *Lab Chip*. 2011; 11:3269–76. [PubMed: 21837324]

Figure 1.

(a) Leukocytes and tumor cells interacting with capture surface proteins under flow conditions. Tumor cells bind stationarily across the entire capture surface, while leukocytes roll on protein mixture functionalized surfaces and detach upon reaching an anti- EpCAM functionalized surface. (b) Polyacrylic acid photopatterned on PDMS channel surfaces left: 2X, right: 20X. (c) Fluorescently labeled anti-EpCAM (red) and E-selectin (green) alternately immobilized on a PDMS channel surface. (d and e) Visualization of MCF-7 (red) and HL-60 (green) cells captured on mixture functionalized surface (d) before and (e) after rinsing with leukocyte elution buffer.

Figure 2.

Surface functionalization process. (a) Photoinitiator (BP) absorbed in the PDMS channel walls is activated, through a photomask by UV radiation thereby initiating graft photopolymerization with the acrylic acid monomers in the channel. (b) PAA is patterned on the PDMS channel surface thereby blocking those regions from silanization with MPTMS. (c) PAA also provides a carboxyl presenting surface to which the protein mixture is attached using a standard EDC/NHS bioconjugation system. (d) Crosslinking reagent GMBS is immobilized to the sulfhydryl groups of MPTMS. (e) Anti-EpCAM is reacted with the NHS ester of the GMBS crosslinker. (f) Resultant pattern of alternating anti-EpCAM and protein mixture. Notice that both attachment chemistries involve amide bond formation between surface carboxyl groups and protein amine groups. The two surfaces are distinguished in that the PAA carboxyl presenting surface requires a soluble crosslinking agent (EDC/NHS) for amide bond formation, while the NHS ester presenting surface has a built-in cross linking agent.

Figure 3. Fraction of MCF-7 or HL-60 cells remaining on protein mixture functionalized capture surface after rinsing with different elution buffers (n = 4). Error bars denote standard error. One way ANOVA: ns not significantly different. *** p < 0.001. * p < 0.05

Figure 4. Patterned surfaces' HL-60 cell capture efficiencies normalized to the PAA immobilized aE-ES surface's HL-60 cell capture efficiency (bars). Fraction of capture surfaces functionalized with E-selectin (diamonds).

Table 1

MCF-7 and HL-60 cell capture efficiency and standard error for patterned and non-patterned surfaces. (n = 3 or 4)

Surface		Homogeneous Suspensions	
		MCF-7 (10^4 mL^{-1})	HL-60 (10^4 mL^{-1})
Immobilization Base	Protein(s) or Pattern Spacing	Mean $\pm$ Std Err (%)	Mean $\pm$ Std Err (%)
Silane	aEpCAM	47.1 $\pm$ 3.8	7.0 $\pm$ 1.3
	aE-ES	79.4 $\pm$ 5.1	30.1 $\pm$ 1.2
	PEG	8.0 $\pm$ 2.3	4.2 $\pm$ 1.7
PAA	aEpCAM	60.6 $\pm$ 5.3	5.5 $\pm$ 0.7
	aE-ES	86.7 $\pm$ 2.0	26.5 $\pm$ 3.2
	PEG	4.9 $\pm$ 0.7	4.7 $\pm$ 1.0
Pattern	Asymmetric	90.1 $\pm$ 4.4	16.1 $\pm$ 2.7
	1000 μm	85.7 $\pm$ 4.6	8.4 $\pm$ 0.9
	600 μm	86.9 $\pm$ 4.3	8.1 $\pm$ 1.0
	300 μm	89.6 $\pm$ 2.5	5.4 $\pm$ 0.6
Cell Mixture Suspensions			
		MCF-7 (10^4 mL^{-1})	HL-60 (10^4 mL^{-1})
Silane	aEpCAM	42.2 $\pm$ 5.6	4.9 $\pm$ 0.8
PAA	aEpCAM	44.9 $\pm$ 4.9	5.0 $\pm$ 1.0
Pattern	300 μm	84.1 $\pm$ 10.3	5.2 $\pm$ 0.6