Bioengineered Surfaces and Hydrogels for Specific Cell Capture and Release from Whole Blood

By

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B.S., Engineering (2006)

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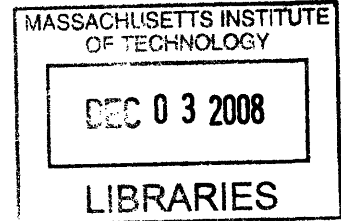
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Submitted to the Department of Mechanical Engineering
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Master of Science in Mechanical Engineering

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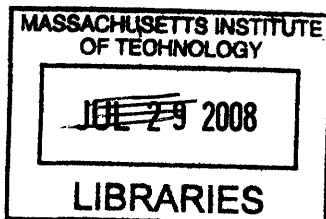
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ABSTRACT

Microfluidic affinity-based cell capture devices are presently able to isolate specific cell populations from heterogenous samples, such as whole blood. The impact of this potentially powerful technology, however, is restricted by the fact that there is no reliable method to release the target cells from the capture surface while preserving their integrity. This work presents the development and evaluation of a functional hydrogel coating that supplements microfluidic capture devices to enable both specific capture and release. The hydrogels are formed by ionically crosslinking a microscale pre-functionalized alginate film on top of the capture substrate. After linking the antibody to the exposed functional sites, the gels may be used to capture cells of interest from physiological solutions. The captured cells may be released by applying a gentle chelating buffer which dissolves the gel, eliminating both the specific and the non-specific cell-surface interactions. This system was evaluated for its ability to capture cells from both buffer and blood. Capture efficiency was found to be equivalent to standard affinity-based devices, and the hydrogel system released 90% of the captured cells without affecting their viability. Finally, the system was validated by capturing and releasing rare circulating tumor cells (CTCs) from the whole blood of a prostate cancer patient; specific immunostaining indicated that the released cells were CTCs based on their expression of cytokeratin and prostate specific antigen. This technology has the promise to significantly influence both clinical diagnostics and basic medical research by enabling rapid enumeration and detailed genetic and phenotypic analysis of rare cell populations.

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CHAPTER 1 INTRODUCTION

1.1 Overview of Microfluidic Cell Isolation

The ability to isolate specific cell populations from complex mixtures such as whole blood has significant utility in both clinical practice and basic medical research. Clinically, enumeration of various cell populations within the blood is essential for making a host of diagnoses. Beyond the apparent hematological analyses which require blood cell enumeration, specific cell counts for unique populations may be used to monitor a variety of other diseases; the most prominent example of this may be current efforts to obtain $CD4^+$ counts as an indicator of disease state in AIDS patients. The ability to isolate specific cell populations is an enabling technology within the research community. Researchers are now able to independently study each cellular component of a pathway of interest. Also, they are beginning to get reliable access to rare cell populations that may be at the center of many questions in human biology; without the ability to isolate these unique cells, their information was previously being drowned out by the high numbers of contaminating cells.

There are a number of approaches to separate cells from a heterogeneous sample. For both bulk and microfluidic approaches, a variety of separation principles are employed including: size based separation, density-based separation, and differential lysis. [1] Each of these principles is based on a physical difference between the target cells and the remainder of the population. Arguably more demanding is the isolation of cells that only unique in their biochemical properties and phenotype. These applications often require exquisite sensitivity and specificity as is afforded by affinity-based techniques. Such techniques capture cells based on cell surface markers that are unique to the target cell population using specific capture moieties; most often

these are antibodies, but may be extended to include any specific binding molecules such as aptamers or selectins. [1,2]

There are two prominent affinity-based bulk-processing techniques for separating cells from a complex sample: fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS). In FACS, the cells of interest are labeled with fluorescent antibodies, optically detected in a flow cytometer, and aerosolized and sorted electrostatically. This process requires extensive sample pre-processing, large volumes of sheath fluid, a relatively high number of target cells as well as dedicated technical staff and expensive equipment. The encapsulation of cells into droplets can also lead to cell damage and phenotypic changes in the cells of interest. MACS mixes antibody decorated magnetic beads with the cell suspension to either positively select the target cells or negatively deplete unwanted cells. As in FACS, MACS requires extensive pre-processing of complex fluids such as blood and is often only a pre-sorting step to enrich rare cell populations, still relying on FACS for the actual cell enumeration and selection. [1]

Continuous flow affinity-based microfluidic devices have the potential to fill an important niche because they can handle large volumes of fluid (mLs), while being able to specifically capture target cells with minimal pre-processing. [3] Depending on the format of the microfluidic device, on-chip analysis of the target population may also be conducted. Microfluidic technologies have been used to scale down FACS approaches, but these technologies often fall victim to many of the same problems are their macroscopic predecessors. [1] Novel label-free immunoaffinity columns have recently been developed which overcome many of the limitations of standard cytometry approaches. [1, 4] These technologies focus on coating a surface with the capture

molecule and then using microfluidics to manipulate the size scale of the device down to one which is on the order of cells, to maximize the cell – ligand interaction. These approaches have shown the ability to isolate rare cell populations while minimizing non-specific capture; also, a label free approach significantly reduces the amount of pre-processing needed, and often detection may be achieved using simple light microscopy to enumerate the target cells, although tagging with fluorescent markers to enable enumeration or phenotyping of specific subpopulations is also feasible. [5] One major limitation of such technologies, however, is the limited ability to recover the captured cells following isolation.

1.2 The Motivation for Cell Release

Microfluidic cell isolation devices have found two major niches: the rapid isolation of clinically relevant cell populations for diagnostics, and the isolation of cells for downstream genetic and phenotypic characterization to help answer fundamental questions in human biology. Within the diagnostics arena, cells are enumerated directly with scanning microscopy or through indirect approaches such as on-chip electrical impedance measurements following cell lysis. [5,6] The ability to release cells following their specific capture would enable simple and direct non-optical detection of the target cell population with much simpler methods, such as the Coulter principle. This has the potential to not only improve the accuracy of target detection, but also could dramatically lower the costs, processing time, and manipulation needed.

A simple cell release mechanism would have even greater implications for more basic biology. While current technologies allow for rapid cell isolation, only limited downstream analysis may be conducted due to the inability to elute the cells from the microchips. This analysis often includes multiplexed immunofluorescence. [7] For genetic analysis, cell populations must be

lysed on chip and extremely careful handling is needed to preserve the small amounts of nucleic acids that may be extracted; additionally, only limited amounts of material may be recovered, restricting the ability to do full genome wide studies. [8] Such approaches do not allow for easy differentiation of unique sub-populations within the often heterogeneous target population. The ability to release and further sort cells could allow for single cell analysis within these rare populations. Thus, the ability to release specifically captured cell populations from microfluidic devices has the potential to be an enabling technology for both personalized medicine and basic cell biology.

1.3 Known Methods for Cell Release and Present Limitations

Current approaches for the release of specifically captured cells range from chemical methods such as gradient elution to mechanical approaches including the use of high shear stress and the use of bubbles within capillary systems. [1,9] Both chemical and mechanical approaches have the potential to cause significant harm to the target cell populations; even if cell viability is preserved, the ability to extract phenotypic and functional information from target populations may be compromised as variations in chemical microenvironments and shear stress are known to cause significant changes in cellular expression patterns. [10] The only known commercial cell sorting system that allows for specific cell capture and release is a MACS based system that tethers the capture antibody to the bead via a proprietary DNA linker and then releases the target cell through the application of DNase to cleave the linker. This approach may be modified for use on capture surfaces, as discussed in section 1.4.

Much work has been done in the field of protein purification, and these techniques would be a natural starting point for developing cell release methods. Protein release strategies, however, generally rely upon the use harsh chemistries including pH extremes and significant variations in temperature or ionic strength. These conditions are not compatible with the desire to elute viable cells. At present, there is no known way to release specifically captured cells bound to a surface that is functional at a physiologic pH, ionic strength and temperature, and does not exert undue chemical or mechanical stresses on the cells of interest.

1.4 Potential Approaches

An examination of the commonly used avidin-biotin chemistry revealed that there are four potential linkages that may be disrupted to enable cell release. As outlined in Figure 1, these linkages are: between the surface and the avidin, the avidin-biotin interaction, the biotin and the antibody, and the antibody and the cell itself. Any alternate chemistry which does not rely upon the use of the avidin-biotin interaction is represented by the generic cleavable linker included in Figure 1.

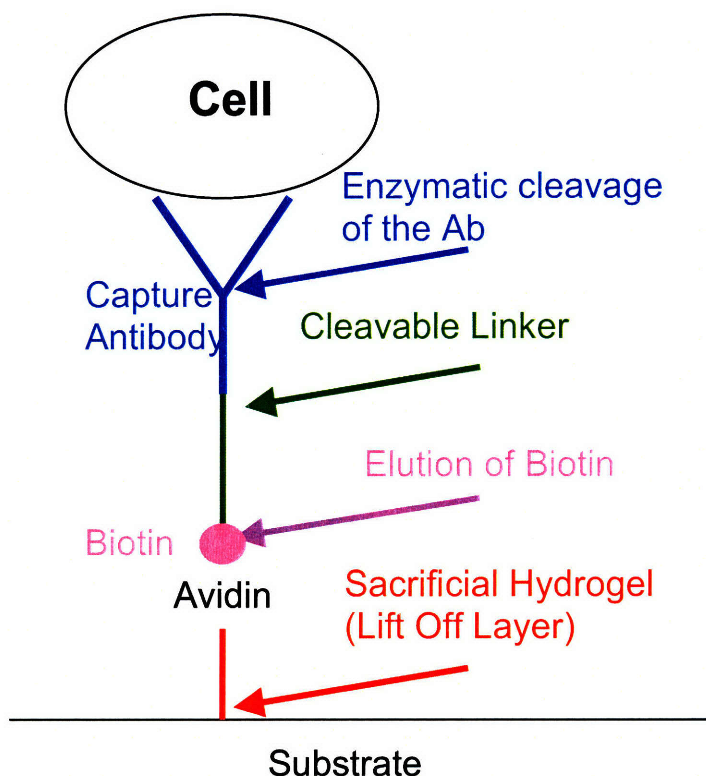

Figure 1 Potential Release Mechanisms

An initial analysis of each of these potential approaches is presented below in Table 1. Of the four approaches

studied, the use of a cleavable linker and the elution of biotin had similar drawbacks as neither of these techniques would be able to cleave the non-specific cell-surface connections, and both would require 100% efficacy to release a cell. The enzymatic approach appeared to have potential, but would likely to harm the cell surface proteins, which are precious for many downstream analyses. The use of a sacrificial layer demonstrated the most promise as it would overcome all of these drawbacks, and had no significant limitations.

Table 1 Pros and Cons of Potential Approaches

Approach	Pros	Cons
Enzymatic cleavage of the capture antibody	Directly frees the cell from any specific linkages. May be combined with other enzymes to free any nonspecific linkages.	Likely to negatively affect cell surface markers. Effectiveness requires precise control of temperature, pH which may not be feasible with an enzyme cocktail.
Insertion of a cleavable linker	Mechanism of degradation may be tunable. DNA linker could be employed.	DNA linker would require application of free DNase, which may affect cells. Will not target nonspecific linkages.
Elution of biotin	Simple competitive interaction.	Would require 100% efficiency to release a cell since there are many tethers. Biotin-avidin affinity is an extremely high.
Sacrificial Hydrogel	Gel degradation properties are tunable. Will directly release both specific and nonspecific linkages. Many tested systems may be adapted for this application.	May affect fluid flow patterns on chip due to gel thickness.

A general scheme for cells captured on a surface via a specific antibody-antigen interaction is outlined in Figure 2. Here, cells will be initially captured via very specific antibody-antigen binding, but may form other non-specific linkages with the surface over time. These non-specific

linkages are likely to confound any molecular mechanism which is focused on cleaving only specific antibody linkages. Because of the variety of non-specific interactions that can occur between the multitude of biomolecules on the surface of a cell and an antibody-coated device surface, we considered the use of a sacrificial layer as a method to simply release both specific and non-specific cell–surface linkages. As an alternative release strategy, enzyme cocktails raise significant concerns with regards to the potential degradation of cell surface proteins and require very closely controlled environments to ensure their efficiency. Development of a functional sacrificial hydrogel coating to enable specific cell capture and release was therefore pursued.

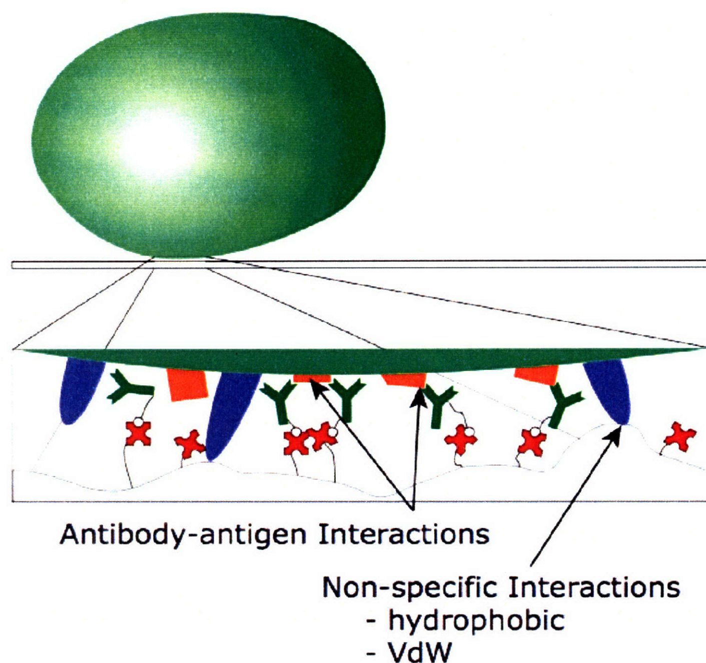

Figure 2 Cell - Surface Interactions

1.5 A Promising Initial Application: The CTC-Chip

Recently, our group has developed a microfluidic affinity-based chip that is able to isolate incredibly rare circulating tumor cells (CTCs) from the whole blood of cancer patients. [7] These cells are believed to disseminate from the tumor, are present in numbers that correlate with patients' clinical courses, and may be involved in metastasis. Therefore, this technology has a significant potential to serve as a diagnostic and prognostic device within the oncology

community, as it would enable a 'blood biopsy'. At present, limited phenotyping and genotyping of these cells is possible because they remain attached to the silicon chip. The ability to release these cells would enable detailed analysis of the CTCs, and aid in our understanding of the metastatic process. Thus, this platform will serve as an ideal testbed for the evaluation of the capture and release approach that will be developed.

1.6 Thesis Outline

Chapter 1 has focused on introducing microfluidic cell isolation techniques, and demonstrating the need for a mechanism by which specifically captured cells may be released from microdevices. Four potential approaches were analyzed, and we concluded that a sacrificial hydrogel layer would best meet the goals of specific cell capture and release without affecting cell viability. Also, the initial test platform, the CTC-chip, was outlined. Chapter 2 presents the development of this hydrogel system. Various spin coating methods, crosslinking techniques and dissolution agents were tested to optimize sub-micron scale gel formation, crosslinking and reversability. Two methods of incorporating biofunctionalization were developed and tested. Gel stability was improved by developing a covalently grafted priming layer. The hydrogel system was then evaluated with cell lines in buffer in Chapter 2, and with cell lines in blood and a clinical patient sample in Chapter 3. Chapter 4 summarizes the major conclusions from this effort and indicates our next steps.

CHAPTER 2: HYDROGELS FOR SPECIFIC CELL CAPTURE AND RELEASE

2.1 Introduction: Challenges in Engineering a Sacrificial Hydrogel

The ideal sacrificial hydrogel material for specific cell capture and release must meet a number of criteria:

- There must be a simple method to form a sub-micron scale coating of the gel on the chip so as not to interfere with the fluid flow patterns engineered into the chip design itself.
- For ease of use and manufacturability, there must be a simple mechanism of reversible gelation.
- The material must have functional groups present that make it possible to couple the specific capture antibody to the gel material itself.
- The material must not promote adhesion of non-specific cells.
- The material must not affect cell viability or phenotype.

The need for a reversible gelation mechanism limits the material specification to only a few specific classes of hydrogels including: photoreversible materials, temperature sensitive materials, pH sensitive materials and ionic hydrogels. Briefly, photoreversible materials were tabled because of the complexities in forming and reversing the gel as well as functionalization; pH sensitive materials were not considered because of the effects of large pH swings on cell viability. Temperature sensitive materials were considered in two groups: UCST (upper critical solution temperature) materials that would gel at room temperature and dissolve at 37°C, and LCST (lower critical solution temperature) materials that would work in the opposite fashion. UCST materials, however, are based on hydrogen bonding associations, and are generally not

stable above a pH of 6.5, so would not be feasible for this application. LCST materials such as pNIPAAM (poly(N-isopropylacrylamide)) are commonly used in cell culture applications, but would require that the chips are continuously maintained at elevated temperatures; this would present significant technical challenges regarding chip storage and processing, as the chip would have to remain heated throughout the cell processing, and the temperature would have to be tightly controlled to ensure cell viability.

2.2 Alginate as a Biomaterial

To best satisfy the criteria outlined above, we selected calcium-alginate, an ionic hydrogel system. Alginate is a naturally derived biomaterial isolated from brown algae and is used across a broad spectrum of applications, from food processing to cell culture. Within the biomedical community, alginate occupies a unique niche due to a number of favorable properties. [11] Alginate is a cytocompatible, non-fouling biomaterial that is generally regarded as safe (GRAS) by the U.S. FDA. A linear polysaccharide, alginate is composed of repeating mannuronic and guluronic acid monomers which form its backbone; this structure contains a readily functionalizable carboxylic acid on each monomer. Alginate is often selected for various applications, most notably cell encapsulation, because of its ability to gently form temperature independent gels via divalent cation (generally calcium) crosslinking under physiologic conditions. [11] This crosslinking is thought to occur due to an 'eggbox' coordination between the divalent ions and the carboxylic acids, but the exact mechanism is not well understood. [12] The gelation is reversible by chelation of the crosslinking cation.

2.3 Materials and Methods

Materials

Standard grade alginate (A2033) was purchased from Sigma-Aldrich (St. Louis, MO); fluorescent beads (G50) were obtained from Duke Scientific (Palo Alto, CA); all cell culture reagents were obtained from Invitrogen Corp (Carlsbad, CA); all chemicals were obtained form Pierce (Rockford, IL) unless otherwise stated.

Cell Culture Methods

All experiments using cell lines used H1650 non small cell lung cancer cell line cells obtained from ATCC. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% Penicillin-Streptomycin at 37°C, 5% CO_2, and were split when flasks were at 70-80% confluence using 0.05% trypsin-EDTA. When pre-labeled cells were used, cells were treated with 10 μM cell tracker orange (Invitrogen Corp.) per manufacturer's instructions.

Statistics and Data Analysis

Each datapoint or result reported in Figures 7, 10, and 11 represent the average of 3 or more independent measurements and each error bar represents the standard error of the mean. Statistical significance was determined by calculating p values using two-tailed students' t-tests assuming unequal variances. To determine linear correlations, r squared correlation coefficients were computed.

2.3.1 Forming Crosslinked Gels and Measuring Their Thickness and Roughness

We explored spin coating methods to deposit thin, uniform coatings of alginate onto the silicon CTC chip. A moderately viscous 2% alginate solution in deionized water was prepared and then carefully dispensed onto the substrate (either a glass slide or the CTC chip, as indicated) until it was fully covered, ensuring that no air bubbles were trapped in the solution. The substrates were then spun at various speeds for 30 seconds and dried.

A variety of methods for crosslinking the films were evaluated; we found that it was necessary to instantaneously crosslink the entire film to prevent it from folding up onto itself, tearing, or otherwise destabilizing. To achieve instantaneous crosslinking, we used an airbrush (H-Set from Paasche Corp., Chicago, IL) and optimized the brush spray pressure (80 PSI) and distance from the sample (8") to produce uniform, micron droplets. By spraying the films with 250 mM $CaCl_2$ in Tris Buffered Saline (25 mM TBS, 150 mM NaCl, pH 7.2) using the airbrush, we were able to instantly and uniformly crosslink the films to form stable hydrogels.

As a confirmation of our ability to coat the CTC chip using this method, 50 nm fluorescent beads were mixed into the alginate solution at a concentration of 0.03% (wt/vol) prior to spin coating several CTC chips. The sample were then crosslinked, trapping the beads within the gels. After multiple washes to remove any loose beads, the gels were then imaged on chip.

To evaluate hydrogel thickness and surface roughness, glass slides were coated with alginate at various spin speeds and then spray crosslinked. Hydrated gel thickness was measured using a

non-contact confocal microscope with materials characterization software (Olympus LEXT OLS3).

2.3.2 Triggering Gel Dissolution

A variety of calcium chelating agents were tested for their ability to reverse the gel. Fluorescent beads were again impregnated into gels formed on glass slides and the fluorescence was measured before and during chelation treatment (t = 5, 10, 20 min). The agents tested were: 50mM EGTA in RPMI 1640 medium, 55 mM Sodium Citrate with 150 mM Sodium Chloride and 30 mM EDTA, 50 mM Sodium Carbonate with 20 mM Citric Acid, and 100 mM EDTA in PBS. 250 mM Calcium Chloride in PBS was used as a control to account for any change in fluorescence with time or exposure. All of these chemicals were obtained from Sigma-Aldrich.

Based on the results presented in section 2.4.2, EGTA in RPMI was selected; the EGTA concentration was then lowered by an order of magnitude to 5 mM and dissolution was evaluated under flow. Glass slides coated with alginate gels were placed into a flow chamber and the EGTA solution was flowed over the slide at shear stresses comparable to those seen in the CTC chip. [7] Details regarding the flow chamber are discussed in reference 13. Finally, cell viability was assessed by adding approximately 10,000 cells per mL to the 5 mM EGTA medium for 2 hours and then checking for viability via trypan blue exclusion and a live/dead fluorescence assay (Invitrogen, L3224) relative to cells kept in RPMI medium alone.

2.3.3 Incorporating Bio-Functionality

The first scheme that was pursued included forming the crosslinked gels and then functionalizing the surface after crosslinking; carbodiimide chemistry was used to link avidin to the surface, as diagramed in Figure 3. EDC was used at a 1:20 molar ratio relative to the number of free uronic acid groups on the alginate, and Sulfo-NHS was used at a 2:1 ratio relative to the EDC; avidin was used at 10 μg / mL and the unbuffered reaction was sustained for 3 hours followed by a 45 minute wash.

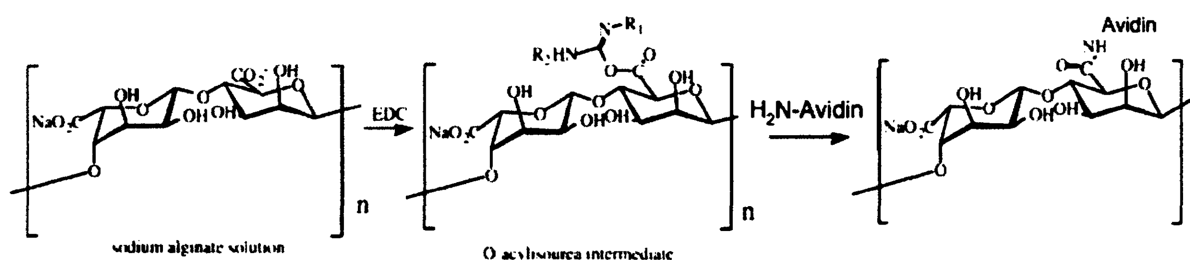

Figure 3 Functionalization scheme to couple avidin to the alginate surface via carbodiimide chemistry

The functionalization was assessed by comparing the fluorescent intensity of FITC-avidin coupled to a gel via EDC to a control sample in which EDC was omitted. The fluorescence of the functionalized gels after EGTA treatment was also measured.

In the bulk functionalization scheme, we used biotin-hydrazide to modify the alginate solution. Biotin hydrazide was mixed with a 1% (w/v) alginate solution at a 1:2 molar ratio relative to the free acid groups. EDC/NHS were used in a 1:2 molar ratio, and the EDC:free acid ratio was varied. Following a three hour reaction in 50 mM MES, pH = 6.5, the alginate was dialyzed (MW cutoff at 10,000) for 72 hours to remove any unbound biotin hydrazide and EDC and then lyophilized. To determine the optimal EDC:alginate ratio, the EDC concentration was varied and

the lyophilized materials were reconstituted to 2% in deionized water, spun coated onto glass slides, crosslinked, and incubated with FITC-avidin for 30 minutes. The chemistry was validated by capturing pre-labeled H1650 cells on the functionalized gels compared to an unfunctionalized control.

2.3.4 Applying Various Substrate Treatments to Improve Gel Stability

In order to study the mechanical stability of alginate gels at the interface of the substrate surface, we varied the surface chemistry of the substrate material. Brief methods for surface functionalization procedures are outlined below. After glass slides were treated as indicated, alginate films were spun on at 3000 RPM and then crosslinked. To test stability, the gels were immersed in 1 mM $CaCl_2$ in TBS and the times to failure were measured.

The different substrate coatings that were tested to improve gel stability can be generally grouped into the following categories: charged surfaces, non-covalent interactions, direct covalent coupling of the gel, and application of a covalently grafted priming layer.

Charged surfaces tested included negatively charged plasma treated slides, positively charged aminosilane treated slides and positively charged poly-l-lysine coated slides (both purchased from Erie Scientific, Portsmouth, NH); organically clean (piranha treated) slides were also tested.

Non-coavlent treatments tested included a carboxylated surface and an avidin functionalized surface. The carboxylated surface was formed by plasma treating piranha cleaned glass and then immersing the slides in 5% 3-(triethoxysilyl)propylsuccinic anhydride (Gelest, Morrisville, PA)

in 95% ethanol at pH 5.0 for 5 minutes which underwent a ring opening to present carboxylic acids. The slides were then rinsed in ethanol and baked at 110°C for 30 minutes. The intent was that the calcium coordination chemistry between carboxylic acids that holds the alginate gel together would also link the gel to the surface. The avidin functionalized surface was tested for its ability to stabilize the biotin-alginate gel via the avidin-biotin interaction. Avidin coated surfaces were prepared as described in reference 7.

Covalent coupling was tested. Gels were formed on epoxy functionalized surfaces; after crosslinking, these gels were treated with 500 mJ of UV energy to form covalent crosslinks between the surface and the gel. Surfaces were epoxy functionalized by applying 3-Glycidoxypropyltrimethoxysilane (Sigma-Aldrich) following the method described for applying the succinic anhydride.

We also explored covalently grafting a priming layer of alginate onto the substrate before spincoating on the gel. The priming layer was grafted by using buffered carbodiimide chemistry to couple alginate to an aminated surface. Key to this reaction, however, was the use of a dilute alginate solution (1 mg / mL), an excess of EDC (3430 EDC:1715 Sulfo-NHS:1 Uronic Acid molar ratio) and a long incubation time (14 hours); these parameters were selected based on a literature review, and are designed to maximize the efficiency of coupling by allowing for alginate chain mobility, having an excess of the crosslinking agent, and letting the reaction continue until almost all of the EDC has been hydrolyzed and is no longer functional. [14] Grafting was verified by washing the slides in deionized water, nitrogen drying them, and then

visually assessing the contact angle. The grafted slides were then dried again and used as substrates for gel formation.

2.3.5 Evaluating the Hydrogel Material

To evaluate the full functionality of the material, we tested its ability to specifically capture and release cells under flow conditions. Functional gels were formed, crosslinked, and dried. Elastomer microchannels, shown in Figure 4 and previously used in our group, were fabricated and clamped on top of these films. [5] To ensure that the clamped system was sealed properly, beads were impregnated in some gels and EGTA was flowed through these systems. As shown in Figure 4, the portion of the gel inside the channel wall dissolved, and the portion outside remained intact, indicating that the liquid seal was secure.

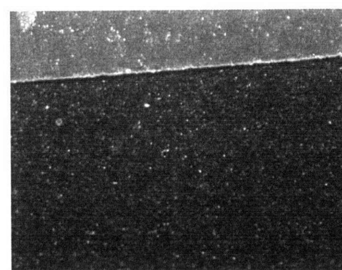

Figure 4 Dissolution of the gel along a microchannel wall, indicating a complete seal in a clamped device.

This system was used to quantify cell capture efficiency and validate cell release. Prior to use, the films were rehydrated with buffer, functionalized with avidin and an anti-human EpCAM antibody as discussed in reference 7. H1650 cells were again fluorescently labeled and spiked at a concentration of 1300 cells per mL into TBS. This cell suspension was then flowed through multiple devices at shear stresses comparable to those found in the CTC chip, and the effluent from the devices was collected so that uncaptured cells could be enumerated. Following capture, the devices were washed to remove unbound cells; this wash fluid was pooled with the effluent. The devices were then either fixed with 1% paraformaldehyde or the cells were released using a release buffer containing 5 mM EGTA.

The capture efficiency was calculated by counting the number of captured cells and dividing by the total number of cells passed through the device. The non-specific binding was measured by excluding the antibody from functionalization and repeating the experiment. The released cells were assayed for cell viability using a fluorescent live/dead assay; a control sample of cells maintained in the capture buffer on a rocker was kept for comparison.

2.4 Results

The results presented below indicate that we are able to form submicron scale gels using spin coating and spray crosslinking techniques (2.4.1). We are also able to dissolve these gels using a gentle chelation buffer (2.4.2). Two schemes to biofunctionalize the gel have been developed, and one has been optimized to saturate the gel surface with avidin (2.4.3). Additionally, a covalently grafted alginate priming layer was developed to stabilize the gel under physiologic conditions (2.4.4). Evaluation of the entire system indicated that the functionalized hydrogels specifically captured target cells and released viable cells (2.4.5).

2.4.1 Thin, Uniform Gels

Spin coating was used to form thin, uniform gels on the CTC chips. To evaluate our ability to coat the chips, fluorescent beads were mixed into the alginate and the chip was coated and imaged; as shown in Figure 5 we are able to completely coat the surface of the chip.

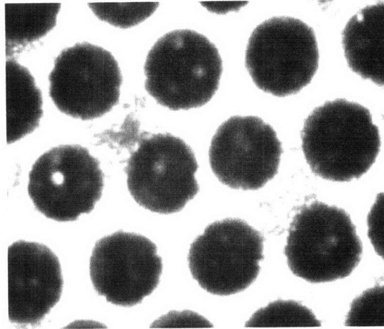

Figure 5 Alginate with fluorescent beads spin coated onto a CTC chip indicating coverage of the chip surface

As the spacing between the posts is as narrow as 30 microns in some locations, it is important that the gels not significantly restrict this gap; any interference would change the fluid flow profile through the chip and could negatively affect cell capture. Gel thickness was studied by varying the spin speed and measuring the thickness. The results are presented in Figure 6. We found a linear correlation between gel thickness and spin speed ($r^2 = 0.94$), as is predicted by spin coating theory. Additionally we observed that the variation in coating thickness decreased with increasing spin speed. Based on these results, we spun all future films at 3000 RPM as this speed produced gels just under a micron in thickness. These gels had an average surface roughness of 37 ± 23 nm (RMS value).

2.4.2 Gel Dissolution

The critical step to releasing captured cells is dissolving the gel. Therefore, multiple chelating buffers were evaluated for their ability and speed to dissolve the gel. Dissolution was measured by impregnating fluorescent beads in the gel, and then measuring the decrease in fluorescence as the beads were released from the dissolving gel (Figure 7). EGTA in RPMI was determined to be the optimal chelating buffer because it had the most rapid dissolution and the medium has the appropriate pH and ionic strength for cell viability. To reduce any potential effects on cell

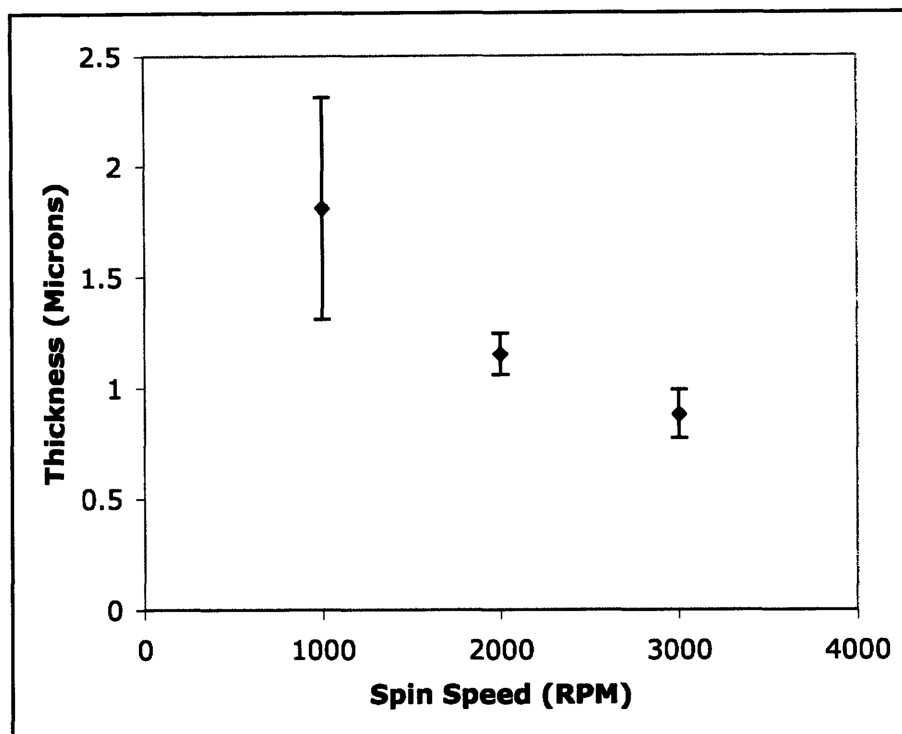

Figure 6 Hydrated gel thickness as a function of spin speed. To form sub-micron scale uniform gels we selected 3000 RPM as our spin speed.

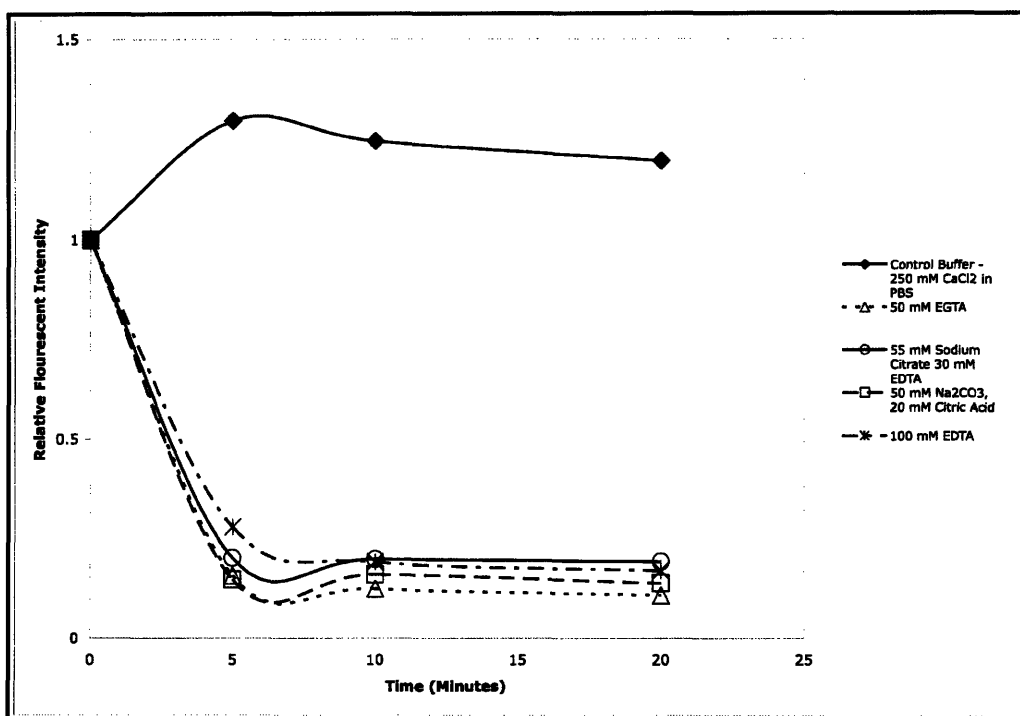

Figure 7 Alginate dissolution rates using various chelating buffers. EGTA in RPMI had the fastest dissolution.

viability due to the chelating agent, the EGTA concentration was lowered to 5 mM and dissolution under flow was confirmed, as shown in Figure 8. At 5 minutes, the fluorescent signal had dropped to 30% of the initial signal, comparable to the results shown in Figure 7.

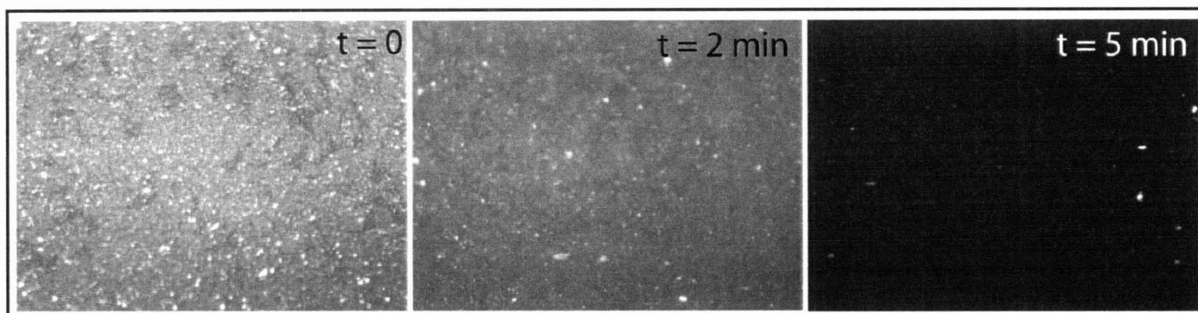

Figure 8 Dissolution of an alginate gel with 5 mM EGTA in RPMI at pH = 7.4 under flow. The fluorescent signal at 5 minutes was comparable to that of the buffers shown in Figure 7.

Finally, cell viability after two hours in the release buffer (5 mM EGTA in RPMI) was evaluated by trypan blue exclusion and a live/dead fluorescent assay; both tests demonstrated that cell viability was not affected.

2.4.3 Bio-Functionalization

Critical to the success of this effort is the ability to reliably functionalize the hydrogels to present the antibody for cell capture. Alginate presents a single carboxyl group per monomer, as discussed in section 2.2. Since this group is readily available for chemical modification, and proteins and antibodies of interest have free amine groups, standard carbodiimide chemistry was pursued. [15] This chemistry has a number of added advantages including being a robust one-step process which requires no protecting/de-protecting, and having no risks of self-crosslinking since only alginate has free carboxyl groups and only the protein or antibody has free amines.

Two functionalization schemes were pursued. In the first, the surface of pre-formed gels were functionalized with avidin. As shown in Figure 9, we were able to functionalize the gel with FITC-avidin using the carbodiimide chemistry. Also, upon dissolution of the gel via EGTA treatment, the functional surface is not present, as fluorescence levels dropped and are now comparable to the control sample.

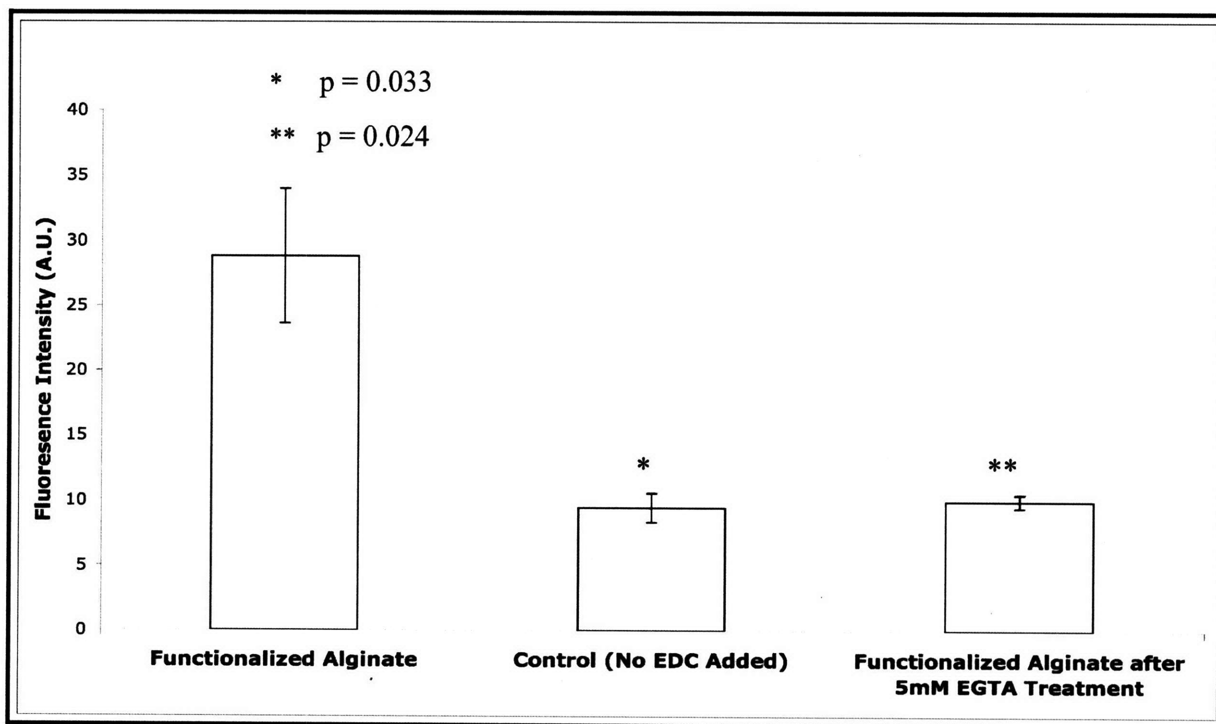

Figure 9 Surface functionalization with FITC-avidin. Specific carbodiimide mediated functionalization was confirmed by comparing to a control sample in which EDC was omitted. EGTA treatment dissolved the functionalized gels, as indicated by the drop in signal intensity.

While this functionalization scheme is sufficient for our application, it would have to be performed individually on each batch of gels. Instead, we sought to develop a highly repeatable and scalable functionalization process. To achieve this, we used the same general carbodiimide scheme, but instead modified the material before spin coating. It would be possible to modify the alginate with avidin in solution, but since much of the material is lost during the coating process, this would be cost-prohibitive. Instead, we coupled biotin to the alginate backbone and

then formed gels with the biotin-alginate material. This scheme was optimized by varying the coupling of biotin to the alginate and then incubating the biotinylated gels with FITC-avidin to determine the amount of biotinylation necessary to saturate the surface of the gel with avidin. As presented in Figure 10 the FITC-avidin signal was saturated at 6.25% activation. To ensure against any potential variability in the chemistry, all future materials were prepared at a 10% activation. As a qualitative check, we were able to capture high numbers of prelabeled cells on the functionalized gel compared to an unfunctionalized control.

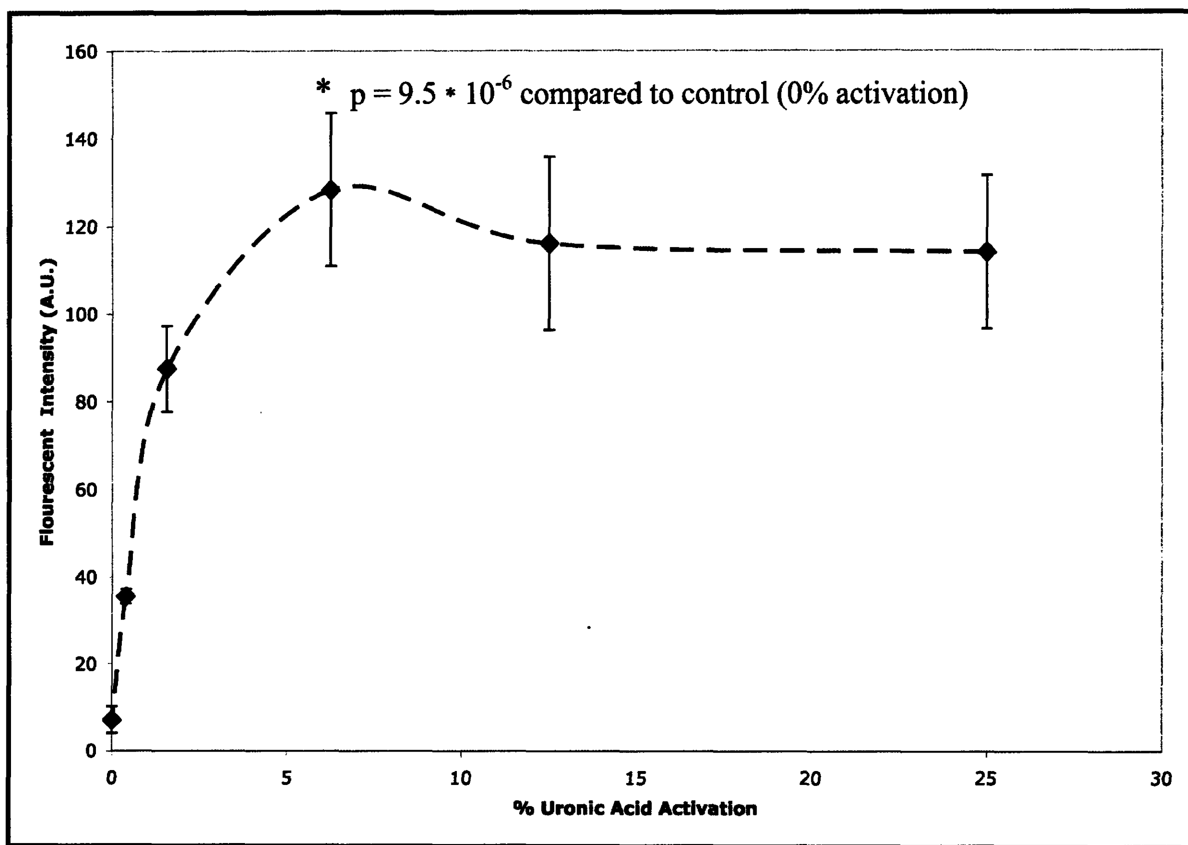

Figure 10 Bulk functionalization optimization. Uronic acid activation was varied by varying the EDC concentration while biotin hydrazide was held in excess. Avidin saturation on gels formed with these materials was achieved at 6.25% activation.

2.4.4 Gel Stability

Table 2 Relevant Ionic Concentrations

Solution	Ca2+ conc (mM)	Ionic Strength (mM)
Standard Buffer	10	150
dH2O	0	0
Physiologic Buffer / Plasma Blood	2 - 2.5	150

To check gel stability in blood, we soaked a gel coated slide in 5 mLs of fresh human plasma isolated from heparin collected blood. Within five minutes, the gel had lifted off of the slide, but was intact and floating in the dish. To understand this further, we considered the ionic components of the solutions presented in Table 2. We found the gels to be stable for over 24 hours when immersed in deionized water. Furthermore, given the fast diffusion of ions in an aqueous solution and the short length scales involved here (sub-micron thickness), Gopferich theory indicates that these gels are bulk eroding, and thus this failure is likely not due to calcium leaching out of the gel at the surface-gel interface. [16] We concluded that this effect was a result of an interfacial failure between the gel and the surface due to competing ionic strength. A number of different surface treatments were considered to improve the surface-gel interaction, as outlined in Table 3. None of the direct ionic, non-covalent or covalent surface modifications tested were able to stabilize the gel-substrate interface for the 4 hours needed to run a CTC cell capture experiment when the gels were immersed in 1 mM $CaCl_2$ in TBS.

Therefore, we tried grafting an alginate priming layer to the surface. Our hypothesis was that a covalently grafted priming layer would be anchored to the surface, and that these alginate chains would be free to interpenetrate with the alginate chains in the gel. Experimentation revealed that grafted slides were very hydrophilic and had contact angles of less than 10°. Both control slides

in which the EDC was omitted from the grafting reaction and aminosilinated slides had contact angles greater than 30°. Gels were formed on these gels and immersed in 1 mM $CaCl_2$ in TBS; they were found to be stable for over 48 hours.

Table 3 Gel Stability on Various Surfaces

Surface Treatment (n ≥ 3)	**Time to failure in 1 mM $CaCl_2$ in TBS**
Piranha cleaned slide	1.5 min
Plasma treated (50W for 35 sec)	< 15 min
Plasma treated (200W for 2 min)	< 15 min
Amine functionalized	100 min
Poly-l-lysine treated	< 70 min
Succinic anhydride (carboxyl) functionalized	< 70 min
Avidin functionalized	< 1 min
Epoxy functionalized + UV	20 min
Surface Grafted Alginate (n = 5)	All samples stable at 48 Hours

2.4.5 Cell Capture, Release and Viability

The full system was tested for the gels' ability to capture and release cells. Gels were formed based on the results presented above, and then dried. A microfluidic channel was clamped on top of the dried film, as detailed in section 2.3.5, and capture efficiency, non-specific binding and released cell viability were measured. The experimental results are tabulated in Table 4.

Table 4 Quantitative Evaluation of Functional Alginate Gel

Capture Efficiency (n =3)	70 ± 2.5%
Non-Specific Binding	7.3 %
Viability of Released Cells (n = 3)	Greater than 90% (Control sample – 86%)

2.5 Conclusions

The data presented above indicates that we have successfully developed a functional, reversible hydrogel system that is able to specifically capture cells under flow and then release these cells without affecting their viability. Microscale gels are formed by spincoating alginate onto the substrate and then uniformly crosslinking the gel with a calcium chloride spray. Gel reversibility was verified, and a dissolution agent was selected, with regard to preserving cell viability. Two different methods to biofunctionalize the gel were developed; a bulk approach was optimized to ensure uniformity and repeatability of the functionalization. A priming layer was developed to stabilize the gel in solutions with physiologically relevant calcium concentrations and ionic strengths. Finally, the system was tested using cell lines in buffers, and was shown to successfully capture and release cells.

CHAPTER 3: EVALUATION OF THE HYDROGEL SYSTEM FOR CAPTURE AND RELEASE FROM WHOLE BLOOD

3.1 Introduction

The hydrogel system under development has been tested in Chapter 2 to show that it meets the design criteria outlined in section 2.1. This chapter focuses on presenting the fabrication process and evaluating the system on the actual CTC chip using both cell lines spiked into whole blood and a blood sample drawn from a prostate cancer patient.

3.2 Materials and Methods

3.2.1 System Design and Fabrication Process

The system consists of a CTC chip grafted with an alginate priming layer and then spin coated with a biotin-alginate hydrogel and functionalized with the EpCAM antibody, as is graphically presented in Figure 11.

The alginate priming layer is grafted onto the silicon CTC chip as presented below. Piranha cleaned chips undergo an oxygen plasma treatment (2% O_2, 50 W, 35 s) and then the surface is immediately aminated by applying a solution of 5% 3-Aminopropyltriethoxysilane, 90% ethanol, 5% deionized water at a pH of 5 for 5 minutes. The chips are rinsed in ethanol, nitrogen dried and baked at 110°C for 30 minutes. They are then immersed in an Alginate/EDC/NHS solution (molar ratios: 1 uronic acid: 3430 EDC : 1715 Sulfo-NHS) with 1 mg / mL alginate in 50 mM MES buffer at pH 6.5. The immersed chips are kept under vacuum for 45 minutes to ensure no bubbles are trapped within the post structure, and then incubated on a rocker for 14 hours, followed by an hour rinse in deionized water. They are then dried with nitrogen.

The biotinylated alginate is constructed by preparing a 1% (w/v) solution of alginate (100 kD) in 50 mM MES buffer at pH 6.5. Biotin hydrazide is mixed in for one hour (molar ratio: 1 uronic acid: 0.2 biotin hydrazide) and EDC and Sulfo-NHS are then added (molar ratio: 1 uronic acid: 0.1 EDC : 0.05 Sulfo-NHS) and the solution is stirred for three hours. The material is then dialyzed (10,000 MW cutoff) for 72 hours against deionized water at 1 mL solution : 60 mL water, changed every 24 hours. The functionalized alginate is then lyophilized and reconstituted at 2% in deionized water.

The gel is formed as follows. The 2% biotin-alginate solution is spun onto the grafted substrate at 3000 RPM for 30 seconds and then air dried. To form crosslinked hydrogels, the films are then spray crosslinked with a solution of 250 mM $CaCl_2$ in TBS using an airbrush at 80 PSI from 8" away. The gels are then rinsed in 2.5 mM $CaCl_2$ in TBS (hereafter simply referred to as buffer), and incubated with 10 μg / mL streptavidin in buffer for 45 minutes and rinsed again. The biotinylated EpCAM antibody is then incubated at 10 μg / mL in buffer for 45 minutes. Finally, the films are rinsed with buffer and nitrogen dried.

All samples are processed under conditions matching those used for standard CTC sample processing, as previously published, unless stated otherwise below. [7] The blood is collected in lithium heparin vacutainers. The wash buffer and base buffer for all other solutions is 2.5 mM $CaCl_2$ in TBS. The release solution is 5 mM EGTA in RPMI 1640, and is run at 10 mLs / hour for 18 minutes, and follows the wash step.

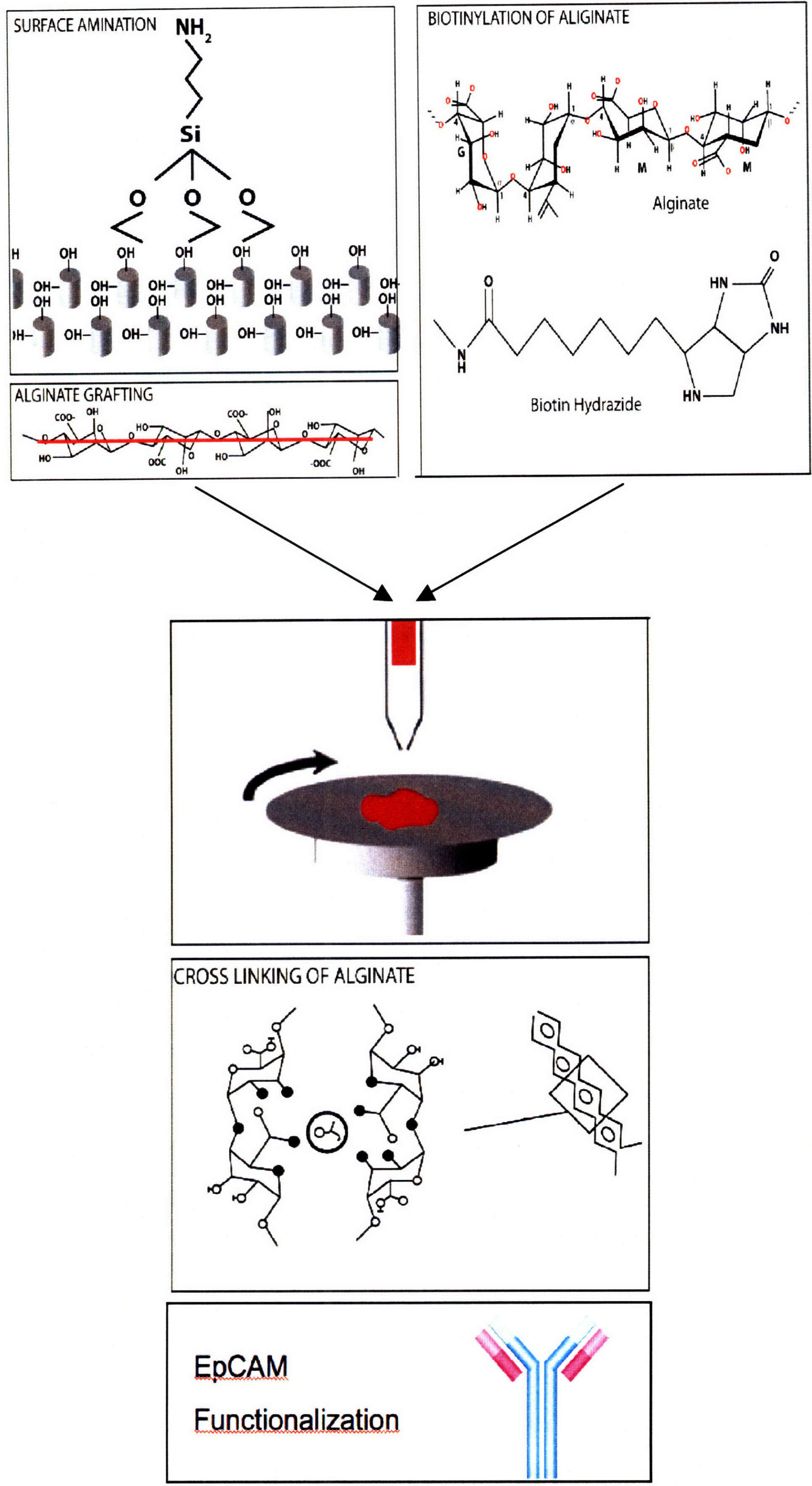

Figure 11 Overview of alginate hydrogel fabrication process

3.2.2 System Evaluation and Validation Methods

The overall cell capture and release system was evaluated by spiking fluorescently labeled H1650 lung cancer cell line cells into whole blood at a concentration of 5000 cells / mL; the cells were prepared as discussed in section 2.3 and all chip preparation and sample processing was conducted as detailed in section 3.2.1. The blood and cells were mixed, and then processed through three chips in parallel:

- A standard CTC chip to quantify the baseline cell capture
- An alginate chip which was fixed following the wash step to evaluate capture on alginate
- An alginate chip from which the captured cells were released following the wash step; this chip was fixed and imaged after 6 mLs of the release buffer flowed through the chip to evaluate the efficiency of the release process

All three chips were stained with a DAPI nuclear stain and imaged for both the specific fluorescent stain and the DAPI stain on a scanning microscope; the entire capture area was included to evaluate cell capture and release.

As a final validation of the cell capture and release system, patient CTCs were captured, released, and immunostained for specific cancer markers. A prostate cancer patient with known metastases was selected as previous visits revealed high CTC counts. Blood was collected in a lithium heparin vacutainer, and processed as previously described. The released cells were imaged immediately following release and then incubated in RPMI medium in a multiwell culture plate overnight to allow the CTCs to attach to the surface. The next day, the well was gently rinsed to remove any unbound cells (presumably the RBCs and leukocytes) and then fixed

and iummunostained for a DAPI nuclear stain and either pan-cytokeratin or prostate specific antigen (PSA).

The pan-cytokeratin staining was conducted as follows (a wash step with PBS was added between each step): the sample was fixed in 4% paraformaldehyde for 1 hour, permeabilized with 0.2% Triton-X for 45 mintues, and stained with a FITC conjugated mouse pan-cytokeratin antibody (Abcam ab11212, Cambridge, MA) used at a concentration of 37.5 μg / mL for one hour and a DAPI nuclear stain (1:1000) for 20 minutes.

The PSA staining was conducted as follows (a wash step with PBS with 10 mM glycine was added between each step): the sample was fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 1% NP40, and then blocked with 3% BSA and 2% goat serum for 30 minutes. The primary polycolonal rabbit anti-human PSA antibody (Dako A0562) was then incubated at a concentration of 3 μg / mL for one hour. The Alexa Fluor 488 labeled goat anti-rabbit secondary antibody (A11008, Invitrogen, Carlsbad, CA) was incubated for one hour at concentration of 2 μg / mL, followed by the DAPI stain (1:1000) for 5 minutes.

3.3 Results

3.3.1 System Evaluation

The system was evaluated by spiking cultured cancer cells into whole blood and capturing and releasing cells using alginate coated CTC chips; the results are presented in Table 5 below.

As shown, the alginate chip has a capture efficiency that is comparable to the control chip. More evaluation would be necessary to determine if this difference is significant; however, this result demonstrates that the addition of a sacrificial alginate hydrogel layer does not appear to affect the interactions between the cell surface and the capture antibody or significantly change the fluidics on the chip.

After cells were released from an alginate coated chip, 10% of the estimated number of captured cells remained on the chip. The solution of released cells contained approximately 3000 cells per mL of blood processed, as counted under fluorescence using a hemocytometer. Together, this data indicates that the release efficiency is 90%.

Table 5 Comparison of Capture and Release Efficiency

	Cells captured per mL of Whole Blood Processed	**Capture Efficiency**	**Release Efficiency**
Control Chip	3291	66%	N/A
Alginate Chip	3609	72%	90%

3.3.2 System Validation Using Patient Sample

Blood from a prostate cancer patient with known high CTC counts was processed and released using an alginate coated CTC chip. The released cells were imaged immediately after coming off of the chip (Figure 12). The granulated cells are the CTCs. After allowing the CTCs to attach to a cell culture treated well, the unbound cells were gently removed and the bound cells were immunostained for cytokeratin and PSA. These results are presented in Figure 13 and 14.

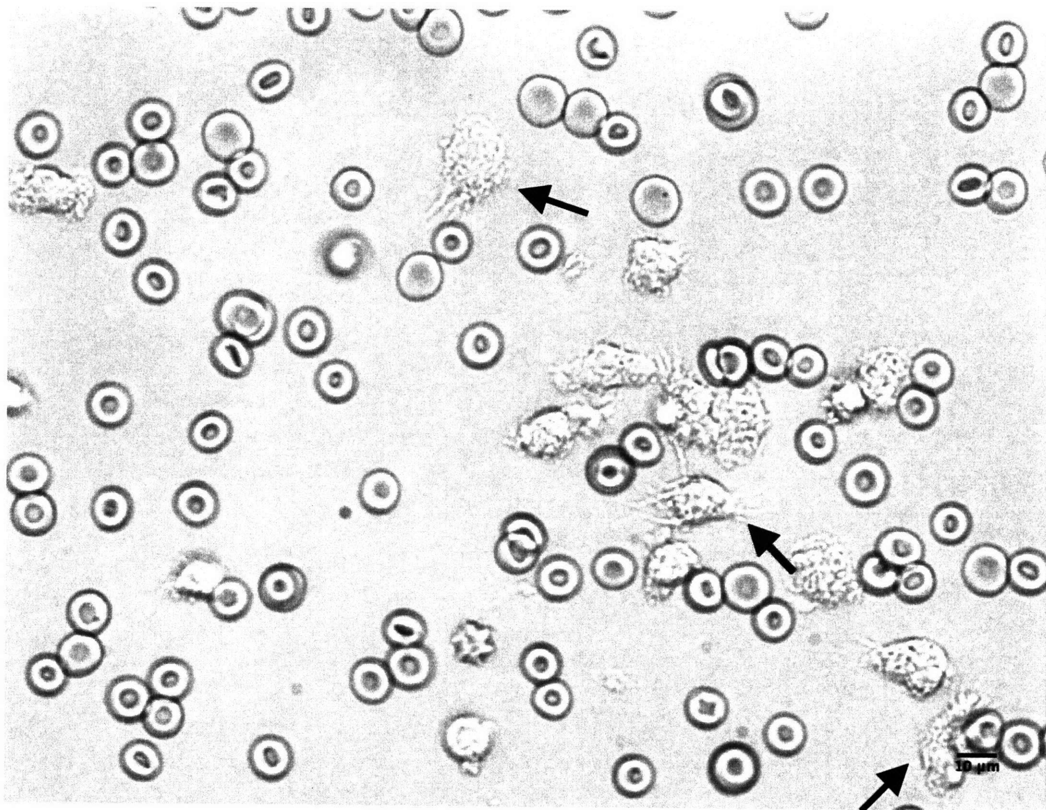

Figure 12 Brightfield image of released patient cells. The granulated cells are CTCs which are beginning to attach to the culture dish (representative cells are marked with arrows).

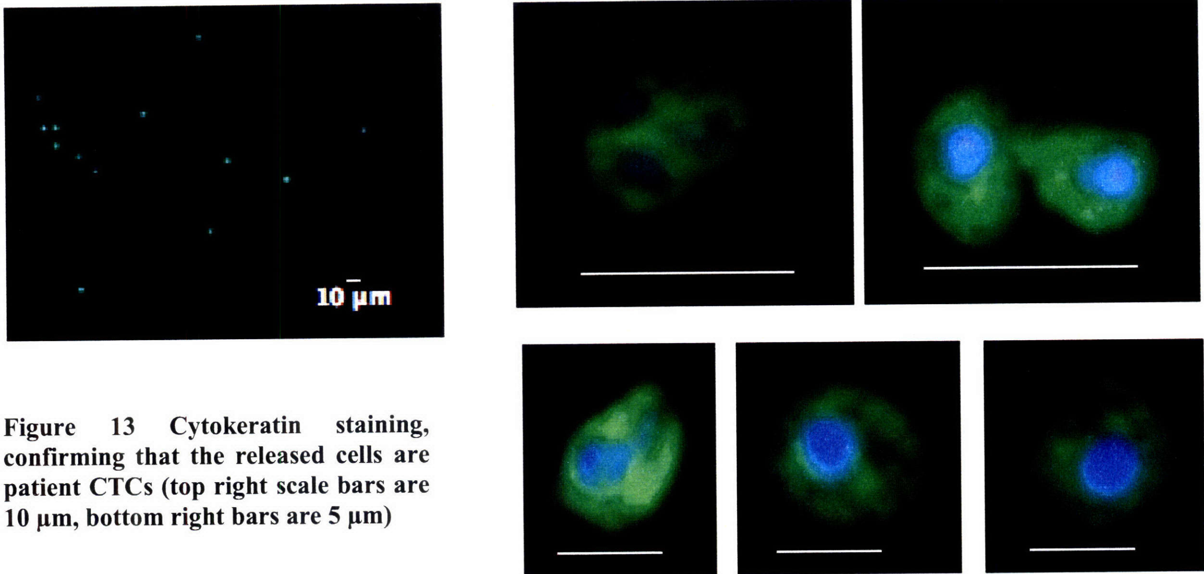

Figure 13 Cytokeratin staining, confirming that the released cells are patient CTCs (top right scale bars are 10 µm, bottom right bars are 5 µm)

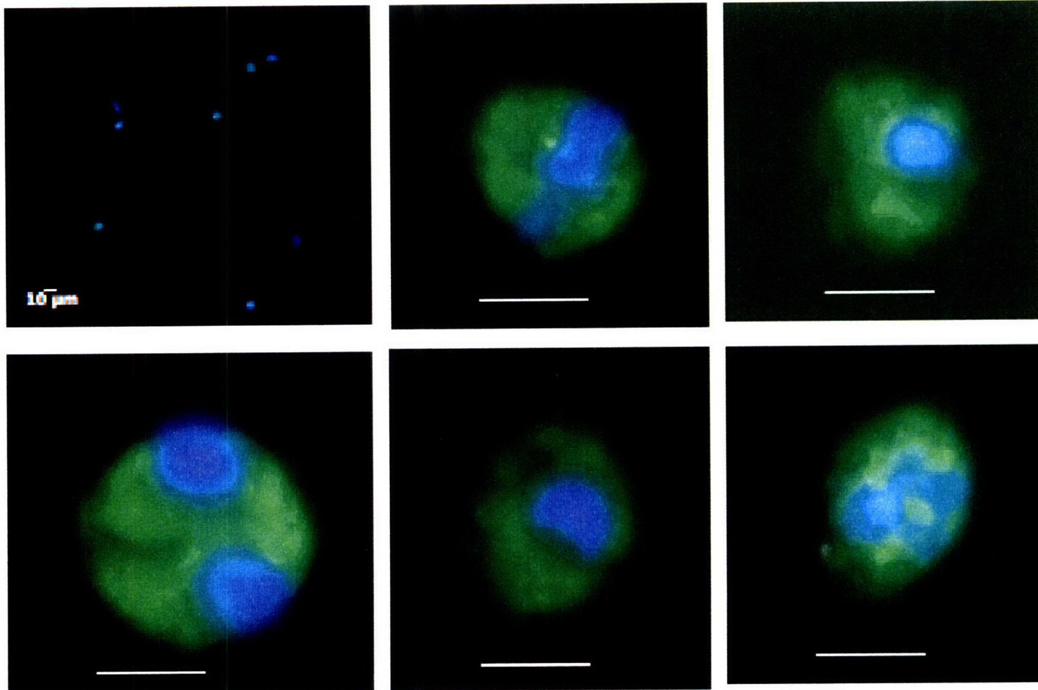

Figure 14 PSA staining of released patient CTCs provide further confirmation. (High mag scale bars are 5 µm)

3.4 Conclusions

This evaluation determined that the capture efficiency from whole blood of the alginate coated chip was at least comparable with that of the standard chip, and the alginate system was able to release 90% of the captured cells. These results affirm that the alginate system is functional and will work well for microfluidic cell capture and release. The patient sample validation further establishes the ability of the hydrogel system to be quickly deployed for this application with confidence. The detection of specific cancer markers at high purity in the released cell population indicates that the specific capture and release process was successful, thereby validating the gel development.

CHAPTER 4: CONCLUSIONS

This effort has been focused on developing reversible hydrogels that may be used for affinity-based cell capture and release. Mircofluidic cell isolation devices have gained recent prominence for their ability to isolate rare cell populations from heterogeneous mixtures such as whole blood. [3] These devices achieve their high specificity of capture by maximizing the cell-surface interaction through careful control of fluid dynamics. While this approach is successful at ensuring cell capture, it also allows the target cells to both adhere to multiple specific ligands and to form many non-specific linkages to the capture surface. As a consequence of these interactions, the captured cells generally cannot be released from the chip in a manner that preserves cellular integrity.

The release of specifically isolated cell populations could significantly enhance the role of microfluidic cell isolation devices in both clinical diagnostics and basic medical research. At present, the cells are able to be rapidly isolated, but since they remain fixed on the chip, only limited downstream analysis may be conducted through on-chip microscopy or cell lysis. If they can be released, cells could be rapidly enumerated and could be far more extensively studied using complex genotyping and phenotyping techniques that are inaccessible with the current design. Therefore, the focus of this work was on developing a means to harness the benefits of microfluidic cell isolation while incorporating a simple mechanism that would allow for the release of unaltered, viable cells following affinity-based capture. To accomplish this goal, we developed a reversible functional hydrogel layer that may be used to coat open microfluidic devices. By incorporating functionality into the gel, the unique fluid flow patterns are preserved,

allowing for cell capture on the gel surface; reversing the gelation eliminates both the specific and non-specific linkages, releasing the captured cells.

We considered a variety of reversible hydrogel materials and selected the alginate-calcium system because it gels independent of temperature, pH, or ionic strength and is reversible by simply chelating the calcium out of the gel. We optimized gel formation and dissolution to form sub-micron gels that are rapidly reversible in near cell culture conditions. We were able to form these gels by spincoating and spray crosslinking, and then dissolve them using a solution of 5 mM EGTA in RPMI medium. Two different methods of incorporating biotin/avidin functionality were evaluated, and we optimized a bulk biotinylation method in which the alginate backbone was prefunctionalized before gel formation. This minimized the variability of the functionalization between chips, and allowed for easy presentation of the capture antibody. To maximize the gels' adhesion to the surface and ensure stability under physiologic conditions, an alginate priming layer was covalently grafted onto the chip.

The entire system was also assembled and tested with both cell lines in buffer (Table 4) and in whole blood. These quantitative assessments indicated that the gel coated devices were able to isolate rare cells with efficiencies comparable to the non-alginate capture chips, and were able to release of 90% of the captured cells without affecting viability. As a final verification, the system was tested on a recently developed microfluidic platform (the CTC-chip) which is being used to isolate rare circulating tumor cells (CTCs) from the whole blood of cancer patients. [7] Gel coated chips were used to isolate these CTCs from a prostate patient's blood; specific capture and release was confirmed by immunostaining the released cells for cytokeratin and prostate

specific antigen. This clinical validation sets the stage for continued use of our hydrogel system to isolate rare cell populations from complex mixtures, and further use of the system to capture and release CTCs is planned. Our ability to study these cells off of the capture chip will enable us to use additional analytical techniques to explore their role in metastasis.

Future efforts to improve this hydrogel system will likely focus on improving the purity of capture from whole blood. At present, the system is limited to using heparinized blood, as heparin does not affect the blood's native calcium concentration. Other commonly used anticoagulants, such as EDTA and sodium citrate, chelate calcium and thus are not compatible with the calcium based gel. Standard affinity-based cell capture devices, however, use these calcium chelating anticoagulants because they significantly decrease non-specific binding compared to heparinized blood. This benefit is due to the fact that calcium is a necessary signaling molecule for cell adhesion. Thus its complete chelation dramatically limits cell attachment to the surface, and increases the purity of specifically captured cells. Any scheme to use these anticoagulants with the present alginate system would require both stabilizing the gel in the presence of calcium chelators, and finding an alternate mechanism of gel reversal. We are beginning to explore one potential method to achieve these requirements. Generally, we envision that the gel would be covalently crosslinked via carbodiimide chemistry with the addition of adipic acid dihydrazide as a crosslinking molecule. The gel could potentially then be reversed by incubation with a specific bacterial enzyme, alginate lyase, that cleaves the polysaccharide into monomer or near monomer units, effectively reversing the gel.

The capacity to release specifically isolated cells has the potential to improve the utility of microfluidic affinity-based cell isolation devices. With the addition of this technology, efforts in diagnostic devices and basic research efforts may be enhanced. Specifically, we will now be able to not only isolate clinically relevant cell populations, but now also be able to rapidly enumerate and functionally analyze them. Also, for the first time, we may be able to develop cell lines from these previously inaccessible rare cells. In conjunction with previous and parallel efforts to develop cell isolation devices, this work provides an enabling technology to further our understanding of the role of rare cell populations in human biology.

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