



Published in final edited form as:

*Anal Chem.* 2012 April 17; 84(8): 3682–3688. doi:10.1021/ac300190j.

## A Biopolymer System for Cell Recovery from Microfluidic Cell Capture Devices

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### Abstract

Microfluidic systems for affinity-based cell isolation have emerged as a promising approach for the isolation of specific cells from complex matrices (i.e., circulating tumor cells in whole blood). However, these technologies remain limited by the lack of reliable methods for the innocuous recovery of surface captured cells. Here, we present a biofunctional sacrificial hydrogel coating for microfluidic chips that enables the highly efficient release of isolated cells (99% ± 1%) following gel dissolution. This covalently crosslinked alginate biopolymer system is stable in a wide variety of physiologic solutions (including EDTA treated whole blood) and may be rapidly degraded via backbone cleavage with alginate lyase. The capture and release of EpCAM expressing cancer cells using this approach was found to have no significant effect on cell viability or proliferative potential and recovered cells were demonstrated to be compatible with downstream immunostaining and FISH analysis.

### INTRODUCTION

Continuous flow affinity-based microfluidic devices are emerging to fill an important niche in cell sorting.<sup>[1,2]</sup> These technologies focus on coating a surface with a capture moiety and then utilize microfluidic architectures to precisely control and maximize cell–ligand interactions.<sup>[3,4,5]</sup> The label-free nature of these techniques enables the isolation of cell populations from complex solutions (i.e., whole blood) with minimal or no pre-processing.

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SUPPORTING INFORMATION PARAGRAPH: Supporting information available includes demonstration of alginate acrylation by NMR, characterization of alginate biotinylation using a modified HABA assay, and equilibrium swelling of gels with varying acrylation.

This allows for the rapid isolation of a wide variety of clinically relevant cell types, ranging from exceedingly rare circulating tumor cells,<sup>[5]</sup> to CD4+ T cells,<sup>[6]</sup> to more prevalent neutrophils.<sup>[7,8]</sup> At present, only limited downstream analysis (most commonly, imaging-based approaches) may be conducted due to the inability to reliably elute viable cells from the microfluidic chips. For genetic analyses, mixed cell populations must be lysed on chip<sup>[9]</sup> and only limited amounts of material can be recovered, restricting the ability to do full genome wide studies of rare cell populations. Furthermore, the cells are unavailable for downstream purification, differentiation of complex sub-populations, single cell genomic analyses, or subsequent culture *in vitro* or in animal models.

Cells initially captured on immuno-affinity substrates via specific antibody-antigen binding are likely to form other non-specific linkages with the surface over time. These non-specific linkages may confound any molecular release mechanisms which cleave only specific antibody linkages. Potential approaches for the release of surface captured cells range from chemical methods such as gradient elution to mechanical approaches such as the application of high shear stress and the use of bubbles within capillary systems.<sup>[10,11]</sup> Both chemical and mechanical approaches have the potential to cause significant harm to the target cell populations. Even if cell integrity 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 gene expression patterns.<sup>[12]</sup>

In limited studies, the combination of a proteolytic enzyme and surfactant enabled the release of captured cells for immediate enumeration<sup>[13-15]</sup>; the degradation of surface markers and potential membrane disruption due to the surfactant, however, may limit the feasibility of this approach for downstream biological analyses of target cells.

Phase-changing hydrogels, such as temperature<sup>[16]</sup> and UV sensitive gels,<sup>[17]</sup> have emerged as a potential method to regulate cell-surface interactions. Recently, Hatch et. al.,<sup>[18]</sup> demonstrated that ionically crosslinked hydrogels formed *in situ* enabled the capture, release, and FACS analysis of endothelial progenitor cells from heparinized whole blood. Notably, this study demonstrated the feasibility of a cation-crosslinked sacrificial hydrogel approach for microfluidic cell capture and release without enzymatic digestion of cell surface proteins. This system, while promising, has a limited scope of use as it cannot be used in conjunction with common anti-coagulation strategies that work on the principle of calcium chelation (EDTA, citrates, etc).<sup>[19,20]</sup> Furthermore, during cell release, target cells are exposed to nonphysiologic levels of calcium chelating agents which may initiate unwanted signaling cascades within the target cells, and have the potential to alter the observed cell phenotype and proliferation state.<sup>[21-23]</sup>

Here we present a photocrosslinked, degradable biopolymer coating that enables the gentle, efficient release of antibody-captured cells from microfluidic devices. Our coatings are of controlled thickness, stable for extended periods of time, and may be used with a wide variety of buffers and physiological fluids (including EDTA-treated whole blood). The release mechanism we employ is the backbone degradation of our alginate biopolymer by a specific bacterial enzyme (alginate lyase) which is commonly used in combination with cell cultures.<sup>[24-26]</sup> We further demonstrate that released cells are viable and proliferative.

## EXPERIMENTAL SECTION

### Alginate Modification

Pharmaceutical grade alginate (Pronova UP MVG, Novamatrix, Norway, 60% guluronate, 40% mannuronate) was modified with both N-(3-Aminopropyl)methacrylamide HCl

(Polysciences 21200-5) and biotin hydrazide (Sigma B7639) using standard carbodiimide chemistry in a single reaction. Briefly, alginate was prepared at 1% by weight in MES buffer, pH = 6.0. Per 100 mL of alginate solution, 0–159 mg of biotin hydrazide, 225.63 mg of methacrylamide, 721 mg of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Pierce 22980), and 408 mg of hydroxysulfosuccinimide (Sulfo-NHS, Pierce 24510) were added and reacted for 3 hours, after which time the solution was dialyzed against dH<sub>2</sub>O for 48 hours and lyophilized. Alginate was reconstituted at 2% in dH<sub>2</sub>O prior to use.

### Hydrogel Formation

Substrates were pre-treated with a molecular-scale layer of alginate by first aminating the surface with a solution of 3-aminopropyltriethoxysilane (Pierce 80370, in 95% Ethanol, pH=5.0 for five minutes) followed by reacting the amine-surface overnight with a dilute alginate solution (0.1% in MES, pH = 6.0) containing 3.73 g of EDC and 2.11 g of Sulfo-NHS per 100 mL. Substrates were then rinsed and dried prior to spincoating. Alginate solutions were spun (spincoater WS-650SZ-6NPP/LITE, Laurel Technologies) at 3000 RPM for 30 seconds to control gel thickness, unless otherwise noted. Gels were then crosslinked using a 250 mM calcium chloride spray, followed by incubation in a 2.5 mM calcium solution, addition of the photoinitiator irgacure 2959 (Ciba Specialty Chemicals) (0.25%) to the solution, and then photocrosslinking in a nitrogen environment for 10 minutes using a 365 nm UV lamp (UVP XX-15-BLB). Following crosslinking, the gels were washed to remove calcium and dried prior to use.

### Patterning and Functionalizing Gels Inside Simple Microfluidic Geometries

Gels were spatially templated onto ultraclean glass slides (Thermo C22-5128-M20) by first applying a laser-cut elastomeric stencil in the shape of the microchannel on top of the glass prior to hydrogel formation. Following gel formation as described above, the stencil was removed, and a PDMS microchannel was plasma treated for 30 seconds (ElectroTechnic Products BD-20) and bonded around the hydrogel.<sup>[6]</sup> The PDMS microchannels used in this study were rectangular chambers 50µm tall, 4mm wide, and 50 mm long, fabricated using standard soft-lithography techniques.<sup>[27]</sup> The channels were flushed with PBS (rehydrating the gels), blocked in a 1% BSA solution for a minimum of 30 minutes (blocking the gel and PDMS walls), and functionalized with neutravidin (Pierce 31000, 50 µg/mL in 1% BSA) for 45 minutes. The channels were rinsed with PBS and incubated with a biotinylated anti-EpCAM antibody (R&D Systems BAF960, 20 µg/mL in 1% BSA for 45 minutes) when used for cell capture. In this model system, only the bottom surface of the channel was coated with the hydrogel, limiting the available area for cell binding.

### Hydrogel Characterization

Hydrogel thickness was measured using a non-contact profilometer (Olympus LEXT OLS3100) after films were formed and dried. To characterize gel dissolution, 50 nm green fluorescent beads (Duke Scientific G50) were mixed into the alginate solution prior to gelation and thus impregnated in the resulting hydrogel; as the gel dissolved, beads were released and cleared away and the decrease in fluorescent signal intensity was monitored using time-lapse imaging. Initial steady-state intensity measurements were taken before treating the gel with alginate lyase at a particular concentration, and a final steady-state measurement was taken once the gel had fully degraded; these values were treated as 100% (initial) and 0% (final) relative intensities. For the control condition (0 µg/mL alginate lyase) all intensities were compared to the initial steady-state, as the gel did not degrade; the slight drop in intensity was observed to be caused by photobleaching of the sample by comparing the intensity of the gel immediately adjacent to exposed field of view. The experimental samples were observed to rapidly degrade, and so no notable photobleaching was observed.

Relative biofunctionality was measured using a sandwich assay in which the biotin incorporated into the hydrogel was coupled with neutravidin, rinsed with PBS, and then followed by a fluorescent biotinylated protein (biotin R-PE, 20 ug/mL for 45 min in 1% BSA, Life Technologies). The 'standard chemistry' is a silane-based coupling chemistry used in our laboratory to functionalize microfluidic devices with neutravidin; it was followed by the same biotin R-PE solution to assay the biotinylated protein binding capacity of the surface.<sup>[1]</sup>

### Cell Capture, Release and Recovery

Cell capture and release was characterized using both a prostate cancer (PC3) and breast cancer (SKBR3) cell line. All cell lines were obtained from ATCC and cultured in accordance with their recommendations.

The relative cell capture efficiency was evaluated by patterning gels in  $10 \times 10$  mm squares and functionalizing with the anti-EpCAM antibody as described. PC3s in PBS buffer were then spotted onto the areas in a static capture assay to compare the relative cell capture potential of the functional alginate coatings as compared to the standard chemistry (positive control, set to 100%) and nonfunctional alginate (negative control). Following a brief incubation period, unbound cells were removed by gently washing the area with PBS. Cells were counted before and after washing to determine capture efficiency. This static assay evaluates the effect of surface ligand presentation on cell capture efficiency, separate from the effects of the microfluidic geometry. Together, these two parameters determine cell capture efficiency in affinity-based microfluidic cell isolation devices.

To evaluate performance of the hydrogel system for cell release and recovery efficiency, a model system was employed; briefly, PC3s were spiked into whole blood ( $10^6$  cells/mL, notably higher than the CTC load found in patient samples) and captured in a microfluidic in which the bottom of the channel was coated with an anti-EpCAM functionalized hydrogel. After PC3s were captured (2 uL/min) and blood was rinsed out with PBS (20 uL/min), the channel was imaged and the total number of cells bound on the gel was counted. Alginate lyase (Sigma A1603, EC# 4.2.2.3, which targets the  $\beta$ -(1-4)-D-mannuronic bonds on the alginante backbone, 1 mg/mL in PBS) was then flowed through the channel (0.5 uL/min), releasing the cells which were recovered in an 8-well chamber slide and counted again. The ratio of cells recovered to cells captured was used to determine the recovery efficiency. The capture areas were re-imaged to confirm recovery efficiency by verifying the mass balance.

Cell release was observed under a fluorescent microscope (Nikon TiE, Japan) by first pre-labeling the cells with a dye (CellTracker Red, Life Technologies). Time-lapse images were taken every 200ms and then analyzed using the tracking module within the manufacturer's software (Nikon Elements) to chart cell movement as a function of time during the release process.

### Analysis of Released Cells

Recovered cell viability was measured using a standard live/dead fluorescent assay (Life Technologies L3224) and compared to control cells which were never introduced into the microfluidic system. Colony formation was measured by recovering PC3 cells from a spiked sample and then diluting the cells with culture medium to form a single cell culture environment. After 96 hours, the number of colonies formed in the well were evaluated alongside the number of colonies formed from a similar number of control cells. HER2 amplified SKBR3 breast cancer cell line cells were captured and released in a similar fashion, cytospun, and then immunostained for the HER2 protein using a primary (Dako rabbit  $\alpha$ -Erb2 A0485) secondary (Alexa Fluor 488 donkey  $\alpha$ -rabbit, Life Technologies)

antibody staining approach. Released SKBR3 cells were also probed with HER2 and centromere FISH probes using standard methods. In brief, released cells were cytopspun and fixed with methanol-acetic acid (3:1), washed with 2X SSC, dehydrated in an ascending series of ethanol, and a HER2/CEP-17 probe mix was added. DNA was then denatured at 75°C, hybridized at 37°C for 20 hours, and post-hybridization washes were performed in 0.4X SSC/0.3% NP-40 at 72°C for 2 min and 2X SSC/0.1% NP-40 at room temp for 30 seconds. The samples were counterstained with mounting medium containing DAPI and imaged at 60X.

## RESULTS AND DISCUSSION

### Hydrogel Development and Characterization

Carboxyl groups on pharmaceutical grade alginate were modified using standard carbodiimide chemistry to present both methacryl groups (65% theoretical derivitization) and biotin moieties. (0–12% theoretical derivitization). (Figure 1) The methacryl groups covalently crosslink the alginate to form a stable hydrogel, and the biotin imparts the bulk material with a ligand for further biofunctionalization. Conjugation was confirmed and quantified using proton NMR and HABA (4'-hydroxyazobenzene-2-carboxylic acid) assays, respectively (SI). As the microfluidic geometry of the channel is critical to maintaining the appropriate shear stress for cell capture, the thickness and roughness of the alginate layer was carefully controlled using spin-coating techniques to produce films in the sub-micron regime that would not affect overall channel fluidics. (Figure 2A) The films were photocrosslinked to form a hydrogel which was stable in the presence of calcium-chelating anticoagulants (i.e., EDTA) but could be rapidly degraded with the addition of alginate lyase (Figure 2B).

To ensure optimal ligand accessibility, a nanopatterning approach was employed similar to that developed by Comisar et. al.<sup>[28,29]</sup> Here, highly biotinylated alginates (86 biotins per chain) were mixed in solution with non-biotinylated ('blank') alginates. These biotinylated alginate chains coil in solution to form nano-islands of functionality spaced apart by blank alginates.<sup>[29]</sup> Optimal island density was studied by varying the ratio of biotinylated chains to blank chains in the copolymer preparation; neutravidin was used to crosslink the gel bound biotins with a biotinylated capture ligand, thereby presenting the capture ligand on the surface. This approach demonstrated an inverse trend in which lower bulk average biotin density in the gel correlated with higher ligand presentation. (Figure 2C) Ligand presentation equivalent to that achieved with the silane-based chemistry commonly employed within microfluidic devices<sup>[1,4]</sup> ("standard chemistry") was realized with 5 to 10 bulk average biotins. (Figure 2C) Static cell capture experiments validated the ligand presentation results; gels functionalized with an anti-EpCAM antibody captured EpCAM expressing prostate cancer cells at a comparable efficiency to that of the standard chemistry. (Figure 2D)

### Release and Characterization of Isolated Cells

To convey the gentle nature of the cell release process, a typical captured cell was imaged during the release process and the position of the cell was tracked over time. (Figure 3) This data demonstrates how, as the gel is degraded, a captured cell (3A) first gradually detaches from the substrate, (3B) then moves slowly along the surface (3C) before being caught up in the flow stream which moves it downstream at the bulk fluid velocity. The efficiency of this cell release process was evaluated by directly quantifying cell capture, release, and recovery. This study demonstrated a  $99\% \pm 1\%$  release efficiency. Released cells were characterized for their viability ( $98.9\% \pm 0.3\%$ ) compared to control cells simply spiked into whole blood ( $99.4\% \pm 0.6\%$ ) and found to be unaffected. (Figure 4A,E) Similarly, effects of the capture

and release process on cell proliferation was studied by diluting released cells in culture medium and measuring the extent of single cell colony formation after 96 hours ( $69.3\% \pm 3.4\%$ ) as compared to similar control cells ( $68.8\% \pm 2.2\%$ ). (Figure 4B,E) As an initial demonstration of the compatibility of the release technology with downstream biological assays, breast cancer cells harboring amplified HER2 genes were spiked into blood, captured, released, and evaluated using standard immunostaining and fluorescence in situ hybridization (FISH) techniques. Expression of the HER2 surface receptor was found to be comparable to control cells ( $113\% \pm 21.2\%$  relative intensity). (Figure 4C,E) Furthermore, HER2 gene amplification is readily evident by FISH, illustrating the potential broad applicability of this cell release technology to enable standard molecular diagnostic applications in a variety of clinical specimens. (Figure 4D)

## CONCLUSION

The alginate biopolymer system presented here represents an important step forward in developing affinity-based cell capture surfaces as it enables gentle, efficient recovery of isolated cells without compromising their viability or proliferative potential. The critical followup of this work is the development of precisely controlled coating techniques for the integration of this materials approach with the complex microfluidic architectures used for rare cell isolation.<sup>[1,4,9,13]</sup> While existing technologies have demonstrated their clinical relevance, recovering these cells with high efficiency and in an unadulterated fashion will place them in the hands of molecular and cell biologists in a manner that is readily compatible with their arsenal of sophisticated tools, so that we may begin to further elucidate the roles of these cells in human biology.<sup>[30,31,32]</sup>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

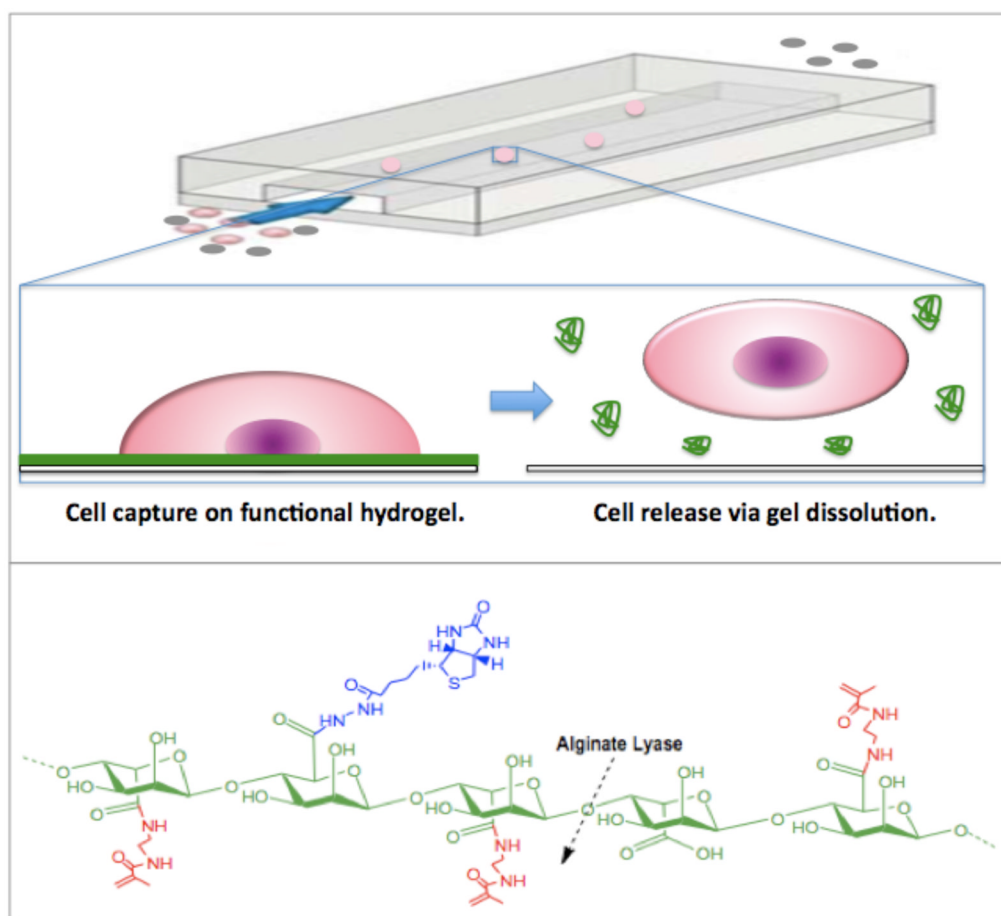
## Acknowledgments

The authors thank Octavio Hurtado and Laura Libby for expert technical support, and Dr. Sangeeta Bhatia for insightful conversations. This work was supported by Dream Team awards from the Prostate Cancer Foundation and Stand Up to Cancer (to D.A.H and M.T) and a Quantum Grant from the National Institute for Biomedical Imaging and Bioengineering (NIBIB) to M.T. A.M.S. is supported by a DOD PCRP pre-doctoral fellowship.

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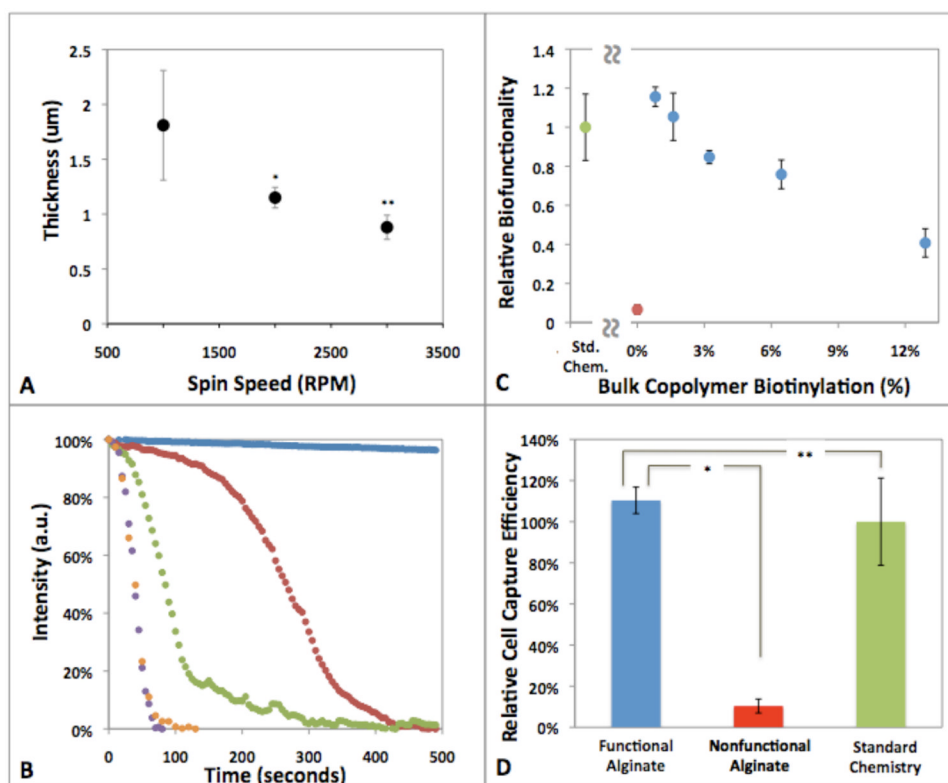
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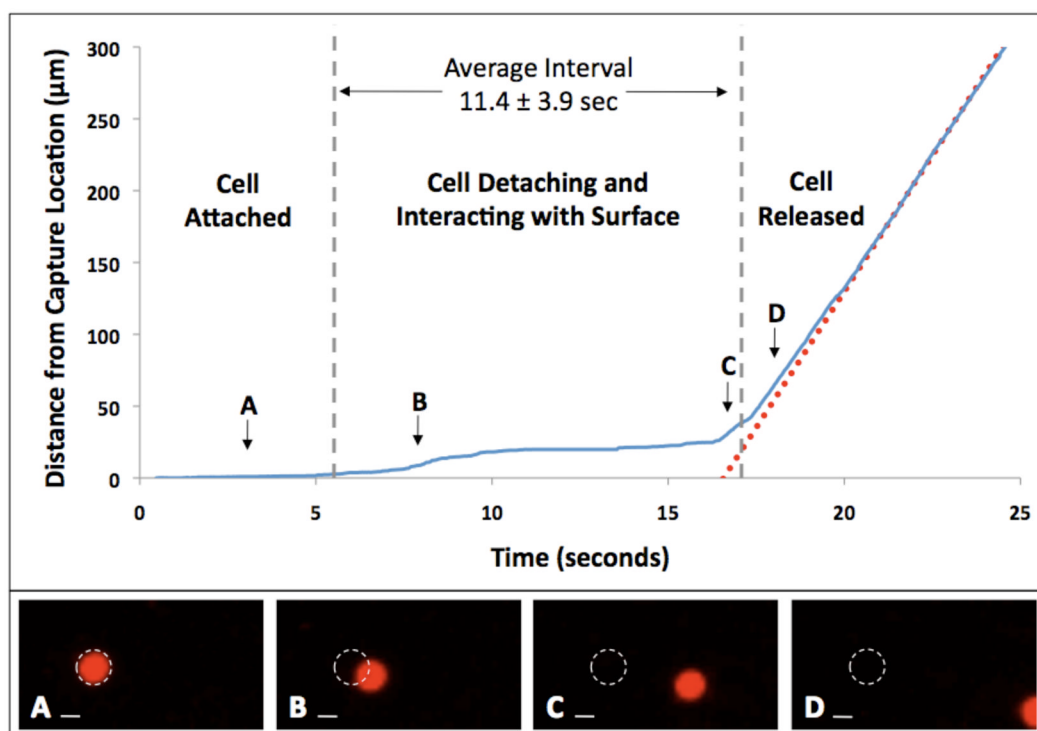
**Figure 1.**

Sacrificial hydrogel coatings may be a compelling strategy for cell release from microfluidic devices, as they will release both specific and non-specific cell-surface linkages. Here, we developed an alginate (green) biopolymer system which may be covalently crosslinked using methacryl groups (red) and biofunctionalized using biotin moieties incorporated in the base material (blue). The gel dissolution and subsequent cell release may be achieved by brief exposure to the bacterial enzyme alginate lyase which cleaves the backbone of the biopolymer.



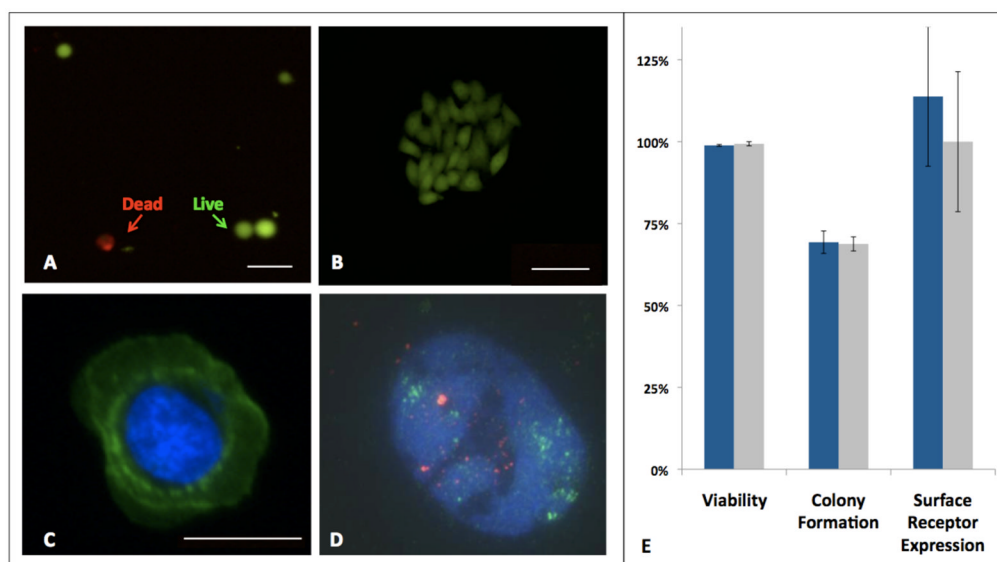


**Figure 2.** Alginate hydrogels were formed with (A) micron-scale thickness using a spincoating process (\*  $p = 0.017$ ; \*\*  $p < 0.001$ ). (B) Upon treatment with alginate lyase at various concentrations (50 (red), 100 (green), 250 (purple), 1000 (orange)  $\mu\text{g}/\text{mL}$ , control PBS (blue)), photocrosslinked hydrogels rapidly degraded in a dose-dependent fashion. Gels were (C) functionalized using gel-bound biotins, and an inverse trend between bulk biotin density and functionality was observed. A static cell capture assay (D) demonstrated that the functional material (blue) captured cells with an efficiency comparable (\* indicates  $p = 0.45$ ) to standard surface modification approaches (green), while non-functional gels (red) resisted physisorption of capture molecules and non-specific cell binding (\*\* indicates  $p < 0.001$ ).



**Figure 3.**

Cells from a prostate cancer cell line were spiked into whole blood, captured on an anti-EpCAM functionalized alginate gel, and released by dissolving the gel with alginate lyase. The progression of a typical cell during the release process (blue) was tracked using automated image processing software. Images A–D show the cell at various stages of the release process and mark the initial location of the cell with a white dashed circle. This series demonstrates the gentle nature of the release process as the cell starts (A) attached, then (B) slowly detaches and (C) travels along the surface until (D) it enters the free flow stream, now traveling at the average bulk velocity of the fluid in the channel (red dotted line). Scale bars are 10 microns. Cells ( $n=15$ ) from 3 different gels were tracked during release to determine the average interval between initial movement to final release.



**Figure 4.**

Released cells were evaluated for (A) viability using a fluorescent LIVE (green)/DEAD (red) assay and (B) colony formation; scale bars are 50  $\mu$ m. Released cells were found to be compatible with downstream (C) immunostaining of cell surface receptors (shown here is HER2 expression in a released cell in green, counterstained with DAPI nuclear staining in blue; 20  $\mu$ m scale bar). (D) FISH analysis, was also feasible as shown here in a released HER2 (green probe) amplified breast cancer cell; the control probe is shown in red. (E) Released cells (blue bars) were found to have comparable viability ( $98.9\% \pm 0.3\%$  vs.  $99.4\% \pm 0.6\%$ ), rates of colony formation from single cells ( $69.3\% \pm 3.4\%$  vs.  $68.8\% \pm 2.2\%$ ), and relative surface receptor expression ( $113.8\% \pm 21.2\%$  vs.  $100\% \pm 21.3\%$ ) when compared to control cells (gray bars) maintained in the appropriate cell culture medium ( $p > 0.05$ ).