Microfluidic Isolation of Endothelial Progenitor Cells for Vascular Tissue Engineering

A Dissertation Presented

by

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Abstract

In the last decade, the regenerative ability of stem and progenitor cells has been demonstrated in several types of tissue. Endothelial progenitor cells (EPCs), which are naturally present in circulating blood, are an especially interesting cell type because they have the ability to repair damaged blood vessels. EPCs have been utilized as precursors in the in vitro cultivation of vascular grafts. As tissue engineering and cell-based therapeutics begin the transition from the laboratory to clinical applications, the availability of robust and simple cell isolation techniques becomes significant. The use of antibody coated channels for cell capture in microfluidic devices has recently been applied to several applications.

The principal goal this thesis is to create microfluidic cell separations systems to isolate or enrich key cell types for tissue engineering applications. In tissue engineering functional cell types must be enriched prior to seeding onto scaffolds. In cell based approaches to tissue repair and regeneration stem and progenitor cells present in certain types must be isolated and characterized prior to use. The adhesion of cells to a functionalized surface is the basis for this type of separation. This work demonstrated the viability of using a PEG-alginate hydrogel for the purification of EPCs. Further it was shown that the coating is agnostic to substrate type. Cells isolated via this hydrogel behaved as expected both in vitro and in vivo. This demonstrates the ability to use this platform for tissue engineering applications, basic research, and cellular based therapies to isolate EPCs in a rapid, rigorous, and inexpensive fashion. This represents a step forward in cell purification methods.
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mixing conditions
1.0 Introduction

In the last decade, the regenerative ability of stem and progenitor cells has been elegantly demonstrated in several types of tissue. Endothelial progenitor cells (EPCs), which are naturally present in circulating blood, are an especially interesting cell type because they have the ability to repair damaged blood vessels. EPCs have been utilized as precursors in the in vitro cultivation of vascular grafts by Mayer and co-workers.\(^1\) The conventional technique of isolating EPCs involves centrifugation (to remove red blood cells and plasma) followed by pre-plating, which involves placing a heterogeneous cell suspension in a culture flask and enricshing the adherent EPCs by successive removal of the supernatant. This method lacks a systematic approach, which introduces variability between isolations and operators. This lack of reproducibility has led to some disagreement about the function and definition of an EPC. It would therefore be beneficial to develop a more reproducible method to help identify and isolate EPCs.

As tissue engineering and cell-based therapeutics begin the transition from the laboratory to clinical applications, the availability of robust and simple cell isolation techniques becomes significant. Microfluidic devices have recently been recognized as effective tools for separation. These devices can be fabricated using soft lithography techniques. This enables devices to be produced en masse in a cost effective and straightforward manner. The channels in these microfluidic devices can range from simple straight flow channels to complex curved channels with expansion and contraction chambers. The microscale geometry of these channels however, can ensure that the flow is laminar regardless of complexity. This laminar flow pattern yields predictable and reproducible results. The primary microfluidic cell separation technologies employed in
this dissertation will be those based on adhesion. The use of antibody-coated channels for
cell capture in microfluidic devices has recently been applied to several applications
including circulations tumor cell capture from blood for cancer, HIV, and sickle cell
diagnostics. \(^{2-4}\) This demonstrates the ability of microfluidic techniques to be scaled up
and used effectively in clinical applications.

The principal goal of my research was to create microfluidic cell separations
systems to isolate and/or enrich endothelial progenitor cells (EPCs) for tissue engineering
applications. In tissue engineering functional cell types must be enriched prior to seeding
onto scaffolds. In cell-based approaches to tissue repair and regeneration, stem and
progenitor cells present in certain types of tissues must be isolated and characterized prior
to use. The use of microfluidic separation schemes for this application has the following
advantages over state of the art methods. First microfluidic techniques are more
systematic. Second, microfluidic systems are capable of isolating rare cells from
heterogeneous suspensions rapidly and inexpensively. Third, recent work has established
design principles for both size and affinity based separations as well as release techniques
for captures cells while retaining function. The adhesion of cells to a functionalized
surface is the basis for this type of separation. By virtue of their micro scale geometries,
microfluidic devices have large surface area to volume ratios; this characteristic makes
them particularly suitable for adhesion based separation processes. Highly specific cell-
ligand interactions have been identified and can be utilized to enact cell separations for
specific subpopulations.

My dissertation focuses on positive selection capture of EPCs from whole blood
samples. Positive selection is a mode of separation in which the desired cell type is
captured using ligands that preferentially bind to it. Captured target EPCs are then released and characterized for their ability to form vascular networks with the ability to form anastomoses, that is, capable of forming vascular networks that will interact with the vascular system of the host they are implanted in.

Adhesion based separations have recently been demonstrated for use in rare cell isolation, using an array of functionalized posts within a microfluidic channel to extract target cells by use of immobilized antibodies. The purpose of the posts in this application is to disrupt the laminar streamlines of the flowing cell suspension. It is further hypothesized that this technique can be used for positive selection of rare progenitor cells from whole blood.

This work has the potential to significantly impact the field of tissue engineering by providing alternative means for isolating EPCs that are more efficient and less costly than existing methods. These cells can then be used in scaffolds to produce vascular tissue with the ability to interact with the host. This will facilitate the implantation of engineered tissues into the host. This advancement will therefore represent a significant step forward in the fields of tissue engineering and regenerative medicine.

1.1.1 Aim 1.1: Design a Capture and Release System

Design a ‘capture and release’ microfluidic cell capture technique to isolate endothelial progenitor cells by positive selection. This aim focuses on the geometric design of the microfluidic device to provide for optimal selection of EPCs and the creation of antibody functionalized alginate hydrogels within the device to release captured cells.
1.1.2 Aim 1.2: Device Geometry and Design

This aim examines the hypothesis that rare stem/progenitor cells captured using the adhesion based positive-selection microfluidic approach can be released with retention of viability and function, and subsequently used in regenerative applications. The ability to capture rare cells using antibody coated pillars in a microfluidic device has been demonstrated by Toner and co-workers. The Murthy group has recently demonstrated how a microfluidic channel coated with a cell adhesive alginate gel can be utilized to capture cells from a flow stream and then subsequently release them with retention of viability and function. This aim combines the design elements of rare cell capture and cell release to effectively isolate stem and progenitor cells resident in tissue niches by taking advantage of their known surface markers and utilize their transformative properties in regenerative medicine applications.

Consideration must be paid to the high viscosity of the hydrogels. The high viscosity will cause a larger pressure drop through the device so care must be taken to avoid rupture. Experiments will be performed to evaluate the feasibility of hand injection of gels into devices of various lengths. The addition of a diluent into the gel to reduce viscosity will also be investigated though this may not be feasible to a large degree without reducing effectiveness of capture.

1.1.3 Aim 1.3: Gel Formulation

Previous work by Plouffe et al. has demonstrated the ability to release cells captured within microfluidic channels by coating the channels with an alginate gel conjugated with the RGDS tetrapeptide. Primary rat cardiac fibroblasts were captured
from a flow stream by this coating. Cell release was accomplished by flowing in EDTA solution and released cells showed minimal loss of viability or function.

Work in this thesis shows that by using similar chemistry, alginate gels can also be conjugated with a variety of capture antibodies including anti CD34 and FLK1. Additionally the impact of incorporating 4-arm amine terminated poly(ethylene glycol) molecules to improve capture efficiency and suppress nonspecific binding is demonstrated.

1.2 Aim 2: Positive Selection of Cells from Human Blood

CD34+ cells were captured directly from human blood in a single device using a cell-adhesive alginate gel coating and released following capture. The device and protocol were used as created in Aim 1. This aim examined the hypothesis that EPCs can be captured from whole ovine blood using the adhesion-based microfluidic approach and effectively released.

For initial proof of concept and optimization studies human blood was spiked with fluorescently labeled EPCs. Following this protocol optimization whole untreated sheep blood will be used. Released cells were analyzed via flow cytometry as well as placed in culture to proliferate.

1.3 Evaluate Function and Phenotypic Identity of Isolated Cells

The objective here was to compare tissue formation (in terms of cell phenotypes and tissue morphology) by EPCs captured by microfluidic separation and EPCs captured by centrifugation and preplating. Captured EPCs were also seeded onto poly(glycerol sebacate) (PGS) scaffolds and incubated for up to 15 days in a roller bottle as described in a recent publication. Tissue formation will be characterized by Western blotting,
histology, and scanning electron microscopy. The objective in this sub-section was to compare tissue formation (in terms of cell phenotypes and tissue morphology) by EPCs captured by microfluidic separation and EPCs captured by centrifugation and preplating.

**1.4 Aim 3: Prepare Platform for Integration into Clinics**

The preliminary experiment performed here were done using a PDMS and glass platform as described in literature. PDMS however is not a suitable material for mass production. Mass production capabilities are essential to getting a technology out of the labrotory and into the clinic. With this in mind, studies were performed to investigate the ability to use alignate gels on a variety of more common commercial grade materials such as polystyrene and polycarbonate. Also, studies were performed to investigate the ability to capture using multiple markers in series. This work increases the relevance of the platform by making the technology more attractive to clinicians.

**2.0 Critical Literature Review**

The isolation of rare cell populations from heterogeneous suspensions such as blood is an essential part of clinical as well as basic research. Clinically, enumeration and analysis of various cell populations within the blood is essential for making a host of diagnoses. Beyond the analyses which require blood cell enumeration, specific cell counts for unique populations may be used to monitor a variety of other diseases; examples of this include current efforts to obtain CD4+ counts as an indicator of disease state in AIDS patients as well as recent studies for monitoring pulmonary hypertension using EPCs as a biomarker. Increasingly in the last decade, miniaturized cell separation devices have been shown to offer many advantages over conventional separation
techniques (e.g. density gradient centrifugation) such as small sample volumes, portability, low cost, improved sterility, and potential for integration with analysis techniques. This review will give an overview of the current cell separation techniques and discuss why there is need to reduce the size of the analyses as well as understand the principle associated with these nascent technologies. First traditional techniques used in the clinic will be described followed by comparing these approaches to the new micro-scale cell isolation methods. Overall this review aims to illustrate the current shortcomings in present cell separation approaches and motivate the need for new and better-developed micro-scale separation technologies.

2.1 Traditional Cell Separation Techniques

Traditional cell separation techniques use the various differences among cells including size, density, expression of cell membrane antigens (antibody-ligand interactions), and osmolarity to selectively isolate target cell types of interest. Several of these techniques suffer from laborious manual sample preparation steps (i.e. density gradient centrifugation, rinsing, lyses and labeling), which not only consume time but can also lead to loss of target cells due to multiple processing steps, inconsistent results and high cost. Additionally these techniques require significant processing volumes which limits their application in clinical environments.

One common modality is the use of specific target antibodies for cell membrane antigens as a way to separate cell populations on the basis of affinity. The most established of these types of separation processes are: fluorescence-activated cell sorting (FACS) and magnet actuated cell sorting (MACS). In FACS, antibodies tagged with
fluorescent dyes are attached to cells in mixed suspensions via receptor-ligand binding. The cells are then sorted individually based on fluorescence and light scattering. Although this technique can provide high purity (95% or higher), it requires expensive equipment (>\$500,000) and has a very limited throughput (\(\sim 10^7\) cells/hour).\(^{11,12}\) Similarly MACS uses antibody-coated magnetic beads along with magnetic collectors as a means of separating target cells populations. This technique allows target cells to be processed in parallel, allowing faster separation (\(\sim 10^{11}\) cells/hour) of high-purity cell populations.\(^{11,12}\) A common element of both FACS and MACS is the need for 30-60 minutes of pre-processing incubation to attach the various tags (fluorescent dyes and magnetic beads, respectively) onto cell surfaces. This step can be avoided by immobilizing antibodies onto surfaces or substrates; this is the principle behind cell-affinity chromatography (CAC). CAC systems can provide a throughput of (10^8-10^9 cells/hour) with yields and purity comparable to FACS and MACS. In general these systems have a packed bed design, which maximizes surface area per unit volume, but also results in long residence times (on the order of 1-2 hours for effective separation).\(^{11,13,14}\) Furthermore, CAC systems are capture based approaches and there currently exist no viable platform for the release of the adhered cells for further analyses and culturing, a key aspect in clinical research.

In addition to affinity-based techniques, several separation techniques involve size-based approaches. These size-based approaches are traditionally utilized in the case of unknown surface markers or large differences in either size of one particular cell type in suspension. A commonly sized-based separation technique is the use of cell strainers.\(^{15-18}\) Here, the cell suspension is separated according to size, where the smaller
cells are allowed to pass through pores of well-defined size where the larger cells then remain in the strainer. This technique is often non-systematic and prone to non-specific cell isolation and a lack of controllable process parameters. Additionally many cell types of interest exist in environments with many other similarly sized cells making separation based solely on size impractical.

Perhaps the most common technique for cell separation is density gradient centrifugation. A density gradient is established along a tube subjected to a centrifugal force. Cells move in the tube under the action of the centrifugal field and stop at the position where their density is equal to the density of the surrounding medium. This technique requires prior knowledge of the density of the target cell (or cells) and a solution capable of generating a density gradient must be used as the medium. Generally several steps are required to effectively isolate one target cell from heterogeneous suspension and density gradient centrifugation is again non-systematic, similar to size-based techniques described previously additionally centrifugation is typically performed in 50 mL tubes, these tubes are prone to nonspecific cell adhesion which results in loss of target cells. Additionally the vast majority of cell types have similar densities making separation by centrifugation impossible.

Three key measures for a separation process are purity, throughput and efficiency, in most cases one is sacrificed for the others rendering an optimal separation impossible. All of the size-and density-based techniques have both a limitation of purity and throughput. In the case of MACS the purity is sufficient, but there is a lack of high throughput. FACS, on the other hand, maintains both high purity and throughput, but requires large expensive equipment and relatively high sample volumes. These inherent
advantages and disadvantages of FACS and MACS-based cell separations illustrate the need for a new and innovative separation technique capable of both the current high throughput and high purity enrichment of conventional instrumentation while being economical and capable of processing low volumes.

2.2 Microfluidic Separation Techniques

Microfluidic devices have recently emerged as effective tools for cell separation. Cells are on the order of microns in diameter and usually handled in aqueous suspensions, micro-scale cell separation offers the advantages of requiring significantly smaller sample volumes versus that required by conventional separation techniques. Additionally, microfluidic devices are portable, low cost and can be easily integrated with numerous downstream analytical techniques (e.g. fluorescence microscopy, PCR, and microarray).

Microfluidic devices can be prototyped rapidly using lithographic techniques developed in the semiconductor industry. The advent of “soft lithography”, a technique for fabricating or replicating structures using elastomeric stamps, molds, and photomasks, resulted in a process whereby an elastomeric polymer (typically poly(dimethylsiloxane), PDMS) can be poured onto a lithographically patterned mold, cured, and then peeled off. The soft lithography process allows for rapid and simple fabrication after the original prototype is created, this patterned polymer can then be bonded directly to glass slides by use of oxygen plasma to create functional fluidic devices. This ability to create replicas of a specific pattern, termed “rapid prototyping”, has been widely recognized for its cost effective and simple nature. This fabrication process allows a range of
microfluidic devices, from simple parallel plate flow chambers to highly complex systems (such as sieves, valves, and even electrical and optical interfaces) to be formed.\textsuperscript{22-30} This versatility allows researchers to go from concept to experiment in a very short period of time without consuming large amounts of resources.

One key advantage to microfluidic cell separation is the ability to vary a variety of parameters easily. The impact of each parameter on metrics such as purity can then be easily assessed. These parameters include flow rate, shear stress and surface composition.

\textbf{2.2.1 Adhesion-Based Microfluidic Separation}

Adhesion-based cell separation systems are akin to chromatography columns whereby a mixture is passed through a column packed with beads or other materials capable of binding to the selected constituents of the feed thus immobilizing these constituents. In the case of cell separation, antibodies immobilized on surfaces are used for binding. An important advantage of this technique is that it may be used to separate cell populations with similar size and/or density, such as subpopulations of human lymphocytes. Another advantage of this approach is that there is no need for pre-processing incubation of the starting cell mixture with fluorescent or magnetic antibody tags, as described earlier. This step is especially important in separations where it is essential to minimize cell activation, which may result in gene expression and phenotype changes\textsuperscript{31}. Macroscale separation designs also minimize residence times inherent in macroscale CAC techniques (on the order of 1-2 hr)\textsuperscript{11, 13, 14}, reducing throughput. Microfluidic CAC systems provide high surface areas per unit volume but their small overall fluid volume keeps residence times short (order of minutes or less)\textsuperscript{32, 33}. 

11
A well known example of adhesion-based microfluidic separation was presented by Toner and co-workers⁵, ³⁴, ³⁵. The authors have developed a microfluidic platform capable of circulating tumor cells (CTC) capture with EpCAM-antibody-coated microposts (Figure 1). The “CTC-chip” successfully identified CTCs in over 99% of cancer patients in a comparable throughput as traditional separation techniques. This device was reported to identify cells in concentrations as low as 5 cells/mL, similar to conventional approaches³⁶.

![Figure 1. Scanning electron micrograph of a capture CTC (pseudo colored red). The inset shows a magnified view of the cell. Adapted from Nagrath et al. [79]](image)

Although a unique and effective tool for CTC identification, the authors recover a low value of ~65% of cells from a known spiked sample and the capture sample was less than
50% pure (i.e. half of the cells captured were not target cells). In addition, the number of target cells captured is determined by fixation with formalin and immunostaining for cytokeratin followed by manually counted by raster scanning the device on a fluorescence microscope. Although this procedure allows for identification of the CTCs versus non-target cells within the chamber, it is cumbersome and ultimately terminates all further live cell experimentation. Therefore, in order to develop an effective monitoring tool for therapeutic use, the recovery and purity must be improved relative to that of traditional methods (FACS and MACS), >95% and >90%, respectively.\textsuperscript{11,12} Also a new enumeration methodology needs be developed that will allow for further cellular experimentation and that does not require the additional fixation and staining steps.

Another adhesion-based technique that was recently developed by Adams \textit{et al.}\textsuperscript{37} uses an EpCAM antibody similar to that employed in the above study but with significant improvements to the purity of the isolated cells. After immunochemical capture, the cells were released via enzymatic digestion and enumerated by a label-free solution conductivity route in which the differences in impedance associated with the CTCs versus blood cells were probed. This method was able to distinguish between cancer cells and blood cells by size and thus improve the purity of the cell count. This method is also much easier, quicker, and more accurate than manual counting. While a unique and interesting approach to improving purity and improving efficiency (97%) versus that developed by Nagrath \textit{et al.}\textsuperscript{5} enzymatic exposure can cause morphological changes due to a disruption of the cell membrane and glycocalyx, leading to losses in cellular activity and viability. Furthermore, enzymatic digestion has been shown to directly affect both the behavior and chemical makeup of the cells themselves.\textsuperscript{38,39} Integration of a platinum
electrode, utilized as the conductance probe in this study, can increase cost and add complexity to the overall design, especially in the case of blood based cell capture methodologies.

Work by Xu et al. 40 developed an adhesion-based device using an aptamer-based approach, in contrast to EpCAM used by prior groups. In this study three different leukemia cell lines in buffer solution were investigated and distinguished from each other. The authors report target cell capture efficiency comparable to that of prior studies (65-97%). 5,34,35,37 In this study the cells were released via mechanical disruption of the aptamer-cell bond and subsequently cultured to validate viability and function; collecting only 47% of the capture cells for culture. This efficiency is too low for a clinical setting or for accurate diagnostics. Also, the effectiveness of the device for whole blood samples still needs to be investigated along with assessment of non-specific binding of healthy leukocytes (a problem encountered by Nagrath et al.)

Another example of an adhesion-based CTC isolation was presented by Cheung et al. 41 who employed anti-N-cadherin as the capture ligand within the device as a means of capturing breast and prostate cancer cells from homogeneous cell suspensions from buffer. This design had the ability to capture ~100% of all the cells in approximately 10 minutes of static incubation. The authors then mechanically detached the cells under various flow rates and accelerations, detaching nearly half of the captured cells with a viability of 80 ± 5%. Similar to the study by Xu et al. 40 this efficiency however, is too low for clinical applications where the target cells are extremely rare. Furthermore, the study was conducted under static conditions in a buffer solution, which is very dissimilar to the complex biological samples from which cells must be obtained. Static capture do
not allow for differentiation of specific binding and non-specific binding due to cellular surface remodeling.

In total, these studies illustrate that for capture only requirements, adhesion-based designs are a viable platform for diagnostics and clinical applications. But most clinical and biological assays required release and collection of the CTCs. Therefore, the shortcomings of these techniques lie in the non-specific binding of the non-target cell populations and difficulty in detachment for further experimentation. These groups have attempted several different enumeration techniques and/or detachment methods with varying degrees of success without achieving comparable separation in comparison to those achieved by traditional isolation technology.

2.3 Release of Captured Cells in Adhesion Based Microfluidics

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 fluorescence microscopy or through indirect approaches such as on-chip electrical impedance measurements following cell lysis. 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 implication for more
basic biology. While current technologies allow for rapid cell isolation, only limited
downstream analysis can currently be conducted due to the inability to elute cells from
the microchips. This analysis often includes multiplexed immunofluorescence. For
genetic analysis cell populations must be lysed on chip and extremely careful handling is
necessary to preserve the integrity of the samples extracted; additionally only small
amounts of material are recovered which restricts the ability to do full genome wide
studies. 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. The
ability to release specifically captured cell populations from microfluidic devices has the
potential to be an enabling technology for both clinical applications and basic cell
biology.

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. 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. The only known commercial cell sorting system that
allows for specific cell capture and release is from Life Technologies 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.

Much work has been done in the field of protein purification, and these techniques would be a great boon to developing cellular release methods. Protein release strategies however generally rely on the use of harsh chemistries such as pH extremes and significant variations in temperature or ionic strength. These conditions are not compatible with the desire to elute viable cells.

Currently there are four general strategies for release of cells from microfluidic devices, these methods are summarized in Table 1.
Table 1: Summary of release mechanisms.

<table>
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<tr>
<th>Method</th>
<th>Advantages</th>
<th>Drawbacks</th>
<th>Ref.</th>
</tr>
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<tr>
<td>Enzymatic cleavage of capture antibody</td>
<td>Can free the cell directly from any specific or non specific binding linkages. Can be combined with other enzymes or techniques.</td>
<td>Likely to affect the cell surface marker. Enzymes have very narrow window of temperature and pH in which they are viable. In the case of stem or progenitor cells the enzyme may cause differentiation or morphological changes.</td>
<td>45</td>
</tr>
<tr>
<td>Insertion of a Cleavable linker</td>
<td>The mechanism may be tunable to be at or near physiological conditions</td>
<td>Targets only specific linkages. Decoupling agent may be harmful to cell.</td>
<td>40, 46</td>
</tr>
<tr>
<td>Shear stress removal</td>
<td>Can target both specific and non specific binding</td>
<td>Shear stress may have detrimental effects on cell vitality or morphology.</td>
<td>47</td>
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<tr>
<td>Sacrificial binding layer</td>
<td>Degradation properties can be tuned by choosing appropriate layer. Can release both specific and non specific binding</td>
<td>May impact flow patterns in certain geometries. Care must be taken to choose a gel with a facile degradation agent.</td>
<td>48-50</td>
</tr>
</tbody>
</table>

Among the several methods of release each has its own benefits and drawbacks. It is necessary to have a convenient, facile release mechanism that does not cause damage to cells or precipitate genotypic changes. For this application alginate is well suited: the release agent is inexpensive and does not damage cells in the time span necessary for release.
2.4 Clinical and Scientific Relevance of EPCs

The identification and characterization of EPCs has been a challenge. There have been two general approaches to identifying, quantifying, and understanding EPCs. First, culture or colony based assays, which rely on the adhesion or lack thereof of EPCs onto culture substrates. Second, the selection of EPCs from heterogeneous mixtures by means of surface markers using techniques such as MACS or FACS. Several subpopulations have been shown in animal models to improve neovascularization and therefore improve the symptoms of ischemia. The precise mechanism and nature of this improvement is still widely unknown. It is therefore necessary to distinguish between these subpopulations in order to discover the mechanism of each. Current methods of isolating such cells all have limitations that prevent them from reliably predicting the behavior in vivo. It is also unknown if such populations exist in vivo or is it they represent an artificial phenotype that is created by removing the cells from the body and culturing them under different conditions.

To further understand EPCs several groups have derived CD34+ and/or CD133+ cells from bone marrow, umbilical cord blood, or peripheral blood. Some researchers have reported difficulties in obtaining colony forming units (CFUs) from peripheral blood.\(^5\) It is possible that these cells may react differently depending on the culture conditions.\(^5\) Additionally it has been found that cultured CD133+ cells may form CFUs with two distinct types of cell colonies, one of small cells with a high ability to proliferate, the second a larger cell colony that exhibits vasculogenic properties.\(^5\)–\(^5\)

Overall many methods produce cells with short-term abilities to improve
neovascularization in animal models. These cells do differ however in their ability to differentiate into endothelial cells and to independently form new vessels. Mature endothelial cells have been shown to form new vessels when implanted into matrigel. However, these results have not been reproduced in animal models.

Besides their therapeutic implications, EPCs have also been studied for as cellular biomarkers for presence of disease such as pulmonary hypertension or as an indication of risk factors for conditions such as cardiovascular disease. The inability to reliably and reproducibly quantify and define these cells however is a hindrance to these methods being adopted as standard diagnostic tools. It seems as though FACS analysis should be the standard methodology for this quantification, however the presence of many short term culture methods used by researches indicates that the field is in need of a new standard. A microfluidic isolation followed by short term culture could add the systematic nature to these cultures that is lacking providing a greater understanding of EPCs their function and their importance.
3.0 DESIGN OF A CAPTURE AND RELEASE SYSTEM

3.1 Abstract

Microfluidic adhesion-based cell separation systems are of interest in clinical and biological applications where small sample volumes must be processed efficiently and rapidly. While the ability to capture rare cells from complex suspensions such as blood using microfluidic systems has been demonstrated, few methods exist for rapid and nondestructive release of the bound cells. Such detachment is critical for applications in tissue engineering and cell-based therapeutics in contrast with diagnostics wherein immunohistochemical, proteomic, and genomic analyses can be carried out by simply lysing captured cells. This chapter demonstrates how the incorporation of four-arm amine-terminated poly(ethylene glycol) (PEG) molecules along with antibodies within alginate hydrogels can enhance the ability of the hydrogels to capture endothelial progenitor cells (EPCs) from whole human blood. The hydrogel coatings are applied conformally onto pillar structures within microfluidic channels and their dissolution with a chelator allows for effective recovery of EPCs following capture.

3.2 Introduction

The use of microfluidic devices in adhesion-based separation of cells is an active area of research in both clinical medicine and basic science. This mode of separation is attractive because no labeling with fluorescent or magnetic tags is needed to drive the separation process, unlike conventional fluorescence- or magnet- activated cell sorting (FACS and MACS, respectively). The high surface area to volume ratios of microfluidic channels together with the ability to enhance surface area with micro-fabricated
structures has enabled such devices to capture cells of extremely low concentrations for a broad range of applications. A major challenge in this area, however, is the lack of methods to achieve nondestructive release of cells captured within microfluidic channels. In a diagnostic context, useful information can be obtained by simple adhered cell counts or by lysing cells on a chip and performing proteomic and/or genomic analysis. However, when isolated cells need to be recovered for therapeutic or scientific purposes, cell detachment must be carried out without causing physical damage and changes in phenotypic identity or function in the cells. These constraints limit the chemical and mechanical forces that can be applied to achieve cell release; for example, enzyme-induced cell detachment is known to cause chemical and phenotypic changes within cells. Furthermore, when simplicity is desired for devices designed for point-of-care and disposable use, the use of electrical, thermal, or optical means of cell detachment becomes infeasible.

In previous work, we have described how alginate hydrogel coatings can be formed on the inner surfaces of microfluidic channels and utilized for cell capture from flowing suspensions followed by release. These coatings contained cell-adhesive molecules covalently bound to the carboxylic acid groups of alginic acid. While these coatings were able to achieve capture and release of primary rat cardiac "fibroblasts from homogeneous suspensions, the adhesion of the cells to alginate hydrogels containing no cell-adhesive molecules was fairly high. High baseline adhesion levels are undesirable when cell capture must be carried out from heterogeneous suspensions of cells, particularly when target cell concentrations are low. As a material, however, alginate hydrogels are easy to create via physical cross-linking in the presence of divalent cations.
and dissolve using relatively low concentrations of chelator molecules, such as ethylenediaminetetraacetic acid (EDTA). In the context of microfluidic devices and as shown in our prior work, these hydrogels can be created by adsorbing functionalized alginic acid within the micro-channels and then forming the gel by flowing a solution of calcium chloride. The concentration of alginic acid in the initial step must be low enough to enable injection into a narrow channel and the flow rate of calcium chloride in the next step must be high enough to ensure that the gel does not fill the entire channel. These parameters can be easily optimized, and the non-covalent nature of the hydrogel micro-channel binding chromatography is relatively new. This paper demonstrates how four-arm, amine-terminated PEG molecules can not only increase the purity of captured EPCs by suppression of nonspecific binding but also enhance capture yield by providing more tether points for capture antibodies.

3.3 Materials and Methods

Microfluidic Cell Capture Device Design. The device uses a post array design similar to that used by Nagrath et al.\textsuperscript{31} To achieve disruption of flow streams and achieve optimal capture, the posts were arranged in a hexagonal layout as described by Gleghorn et al.\textsuperscript{6} The posts have a diameter of 100 \( \mu \)m and a transverse spacing of 150 \( \mu \)m from center to center. Rows have a center-to-center spacing of 125 \( \mu \)m and each is offset by 50 \( \mu \)m. The post array is 0.7 cm long and 0.5 cm wide. The posts height was approximately 50 \( \mu \)m for the devices fabricated by soft lithography as described below.

**Microfluidic Device Fabrication.** A 2-dimensional projection of the cell capture device was drawn using AutoCAD in-house, and the image was imprinted at high resolution onto a chrome mask by FineLine Imaging (Colorado Springs, CO). This
photomask was utilized to generate a negative master at the George J. Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University. In précis, a silicon wafer was coated with SU 8-50 photoresist to a thickness of approximately 50 µm. With the mark overlaid, the wafer was exposed to 365 nm, 11 mW/cm$^2$ ultraviolet light from a Q2001 mask aligner (Quintel Co, San Jose, CA). Unexposed photoresist was then removed using SU 8 developer.

For poly(dimethyl siloxane) (PDMS) device fabrication, the silicone elastomer and curing agent were mixed in a 10:1 (w/w) ratio and poured on top of the negative master wafers, degassed, and allowed to cure overnight at 65 °C. PDMS replicas were then pulled off the wafers prior to punching inlet and outlet holes with a 19-gauge blunt-nose needle. The replicas and glass slides were exposed to oxygen plasma (100 mW with 8% oxygen for 30 s) in a PX-250 plasma chamber (March Instruments, Concord, MA) and then immediately placed in contact with each other. The irreversible bonding between PDMS and glass was completed by baking for 5 min at 65 °C.

**PEG/Antibody-Functionalized Hydrogel Synthesis.** Seven different hydrogel formulations were investigated in this study and these are designated as Gel Types I-VII. For Gel Type I, 45 mg of alginic acid, 4.8 mg EDC, 13.2 mg sulfo-NHS, and 20 µL inert IgG (1 g/mL) were added to 2 ml of MES buffer solution and mixed using an IKA Ultra Turrax Tube Disperser for 29 min and allowed to incubate for 60 min. For Gel Type II, 45 mg of alginic acid, 4.8 mg EDC, 13.2 mg sulfo-NHS and 100 µL anti-human CD34 (200 µg/mL) were added to 2 mL of MES buffer, mixed as before, and incubated for 60 min. For Gel Type III, 45 mg alginic acid, 4.8 mg EDC, 13.2 mg sulfo-NHS, 45 mg 20k MW PEG, and 100 µL anti-human CD34 were added to 2 mL of MES buffer, mixed for
29 min, and allowed to incubate for 60 min. Gel type IV consisted of 45 mg alginic acid, 4.8 mg EDC, 13.2 mg sulfo-NHS, 22.5 mg 10k MW PEG, and 100 µL anti-human CD34 added to 2 mL of MES buffer, mixed for 29 min and allowed to incubate for 60 min. Gel Type V was created by mixing 4.8 mg EDC, 13.2 mg sulfo-NHS, 22.5 mg 10k MW PEG, and 100 µL anti-human CD34 in 2 ml of MES buffer for 29 min and then adding 45 mg of alginic acid followed by 29 min of mixing and 60 min of incubation. Gels VI and VII were formed by mixing 22.5 mg 10k MW PEG with 100 µL antibody in 2 mL of MES buffer and mixing for 10 min and 29 min, respectively, and incubating for an additional 15 min and 60 min, respectively. 4.8 mg EDC, 13.2 mg sulfo-NHS, and 45 mg alginic acid were then added to the mixture, mixed for 29 min and allowed to incubate for 60 min.

Following the incubation step, each functionalized alginic acid solution for each gel type was injected into a Slide-A-Lyzer Dialysis Cassette 10,000 molecular weight cut-off (Fisher) and dialyzed against MES buffer for 48 hours to remove unreacted sulfo-NHS and EDC. Table 2 summarizes the synthetic steps and components for each gel type. Steps 1 and 2 indicate the sequential nature of the protocol followed for combining the respective reagents.
Table 2. Summary of seven gel types studied and mixing conditions for each.

<table>
<thead>
<tr>
<th>Gel Type</th>
<th>PEG MW [kDa]</th>
<th>Components in Each Mixing Sequence†</th>
<th>Mixing/Incubation Times [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alginic Acid  EDC &amp; Sulfo-NHS  PEG Antibody*</td>
<td>Step 1</td>
</tr>
<tr>
<td>I</td>
<td>none</td>
<td>1 1 - 1</td>
<td>29/60</td>
</tr>
<tr>
<td>II</td>
<td>none</td>
<td>1 1 - 1</td>
<td>29/60</td>
</tr>
<tr>
<td>III</td>
<td>20</td>
<td>1 1 1 1</td>
<td>29/60</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>1 1 1 1</td>
<td>29/60</td>
</tr>
<tr>
<td>V</td>
<td>10</td>
<td>2 1 1 1</td>
<td>29/0  29/60</td>
</tr>
<tr>
<td>VI</td>
<td>10</td>
<td>2 2 1 1</td>
<td>10/15 29/60</td>
</tr>
<tr>
<td>VII</td>
<td>10</td>
<td>2 2 1 1</td>
<td>29/60 29/60</td>
</tr>
</tbody>
</table>

† “1” denotes reagent added in step 1; “2” denotes reagent added in step 2.
* Inert IgG was used for Gel Type I; anti-human CD34 was used in all other gel types.

**Infrared Spectroscopy.** Functionalized alginic acid samples were spread on poly(tetrafluoroethylene) (PTFE) sample cards (Crystal Labs, Garfield, NJ) using a spatula and allowed to thicken for 4 h. The cards were then inserted into a Perkin Elmer 1000 Fourier-transform Infrared (FTIR) spectrometer. The absorbance at 638 cm⁻¹, corresponding to amide groups, was analyzed and compared for each gel type.

**In Situ Hydrogel Formation within Microfluidic Devices.** A 1 g/mL solution of CaCl₂ in deionized water was injected into each device (by hand, using a 1 mL syringe)
and allowed to incubate overnight. The CaCl$_2$ solution was then withdrawn by hand using a 1 mL syringe. The PEG- and antibody-functionalized alginate solution prepared for each gel type was then injected into the devices by hand and allowed to adsorb for 1 h. Next, the devices were rinsed with MES buffer at 10µl/min for 10 min using a Harvard Apparatus PHD 2000 syringe pump (Holliston, MA), followed by a 100 mM CaCl$_2$ solution in MES buffer at 10 µl/min for 10 min to form a thin layer of hydrogel on the walls of the microchannels. Finally, the devices were rinsed with MES buffer at 5 µl/min for 10 min to remove unreacted CaCl$_2$.

**BCA Protein Assay.** A BCA protein assay solution was prepared according to manufacturer instructions. The solution was then injected into each device at 5 µl/min for 40 min. The output was collected in a microplate and absorption at 562 nm was measured using a Bio-Tek Powerwave XS spectrometer.

**Blood Draw.** Whole human blood was drawn from healthy volunteers in heparin collection tubes under a protocol approved by the Northeastern University Institutional Review Board.

**EPC Capture Protocol.** Whole blood was injected into microfluidic capture devices at 5 µL/min for 60 min. Each device was then rinsed with MES buffer at 10 µL/min for 5 min. For release of captured cells, a 50 mM solution of EDTA in PBS was injected at 10 µL/min for 10 minutes and the output was collected in a 1.5 mL microcentrifuge tube. Each individual experiment included 10 microfluidic devices. 300 µL of blood was passed through each device, at the rate specified above. The cells released from each device were pooled into a single suspension to allow enumeration by flow cytometry. Hence the data reported in Figure 2a-b represent yield and purity for
Figure 2. Yield (a) and purity (b) of EPCs captured from whole blood within microfluidic devices coated with PEG- and antibody-functionalized hydrogels. 0.3 mL of whole blood collected in heparin tubes was directly injected into individual microfluidic devices and 10 devices were run in parallel. Cells released from each device were pooled into a single suspension to allow enumeration by flow cytometry. Data reported in (a) and (b) represent yield and purity for EPCs recovered from a total blood volume of 3 mL. Error bars denote standard deviations based on 3 independent measurements of EPC and total cell counts made with the same sample. For EPC enumeration, cells released from each device were mixed with 10 µL each of anti-human CD133 PE, anti-human CD44 FITC, anti-goat FLK-1, and anti-goat IgG PerCP. The mixture was stored in the dark for 30 min and centrifuged at 130 × g for 10 min. The supernatant was decanted and cells were suspended in 200 µL of PBS for enumeration using a Beckman Coulter Cell Lab Quanta SC flow cytometer. While there is some debate as to the
precise surface marker profile of EPCs, a general definition based on a number of literature sources was applied in the present study and cells that were CD133+, CD45-, and FLK-1+ were counted as EPCs.\textsuperscript{16-20}

3.4 Results and Discussion

Figure 3 shows infrared (IR) spectroscopy data for the functionalized alginic acid solutions emerging from the one- or two-step synthesis protocol. The spectra for gel types II-VII show equivalent levels of amide group content. In all of these gels, amide groups can result not only from binding between antibody and PEG molecules but also from antibody-alginic acid and PEG-alginic acid binding. The IR spectroscopy analysis therefore reflects that antibody and amide bond content, taken together, is very similar across all gel types, indicating successful incorporation of antibody and PEG; however no functional distinctions between the different gel types can be predicted from these data. Figure 4 shows relative total protein measurements made using a BCA assay kit. The BCA solution becomes more transparent as it comes in contact with proteins such as antibodies. Hence, by flowing this solution through hydrogel-coated microfluidic devices, the amount of accessible antibody on each gel type can be compared. The protein content of the solutions exiting the devices is shown as a function of gel type in Figure 4 and is expressed in arbitrary units of absorbance rather than as a calibrated mass or concentration. Such a calibration cannot be performed as the interaction between the BCA solution and protein is a flow-surface interaction whereas standard calibrations
would be carried out by mixing and incubating together the BCA solution and a solution of known protein concentration. However, the relative measurement nevertheless allows comparison of the accessible anti-CD34 capture antibody between each gel type. Figure 4 shows an increase in accessible antibody from gel types I-VII while the total antibody and amide content of the mixture remains constant (Figure 2), indicating an increase in the efficiency of conjugation between the gelled surface and the antibody.

Figure 3. Infrared spectra of PEG- and antibody-functionalized hydrogels (gel types II-VII; not normalized to avoid total overlap) compared to standard solutions of anti-CD34 with concentrations of 0.1 mg/mL and 0.05 mg/mL. The relative intensities of the hydrogel sample peaks are comparable, indicating similar levels of antibody-loading. Note that this measurement is a bulk measurement that does not distinguish between covalently-bound antibody from antibody molecules that are physically trapped within the hydrogels.
Figure 4. Qualitative measurement of accessible antibody within hydrogel-coated microfluidic devices. A bicinchoninic acid (BCA) assay kit was utilized to measure the relative amount of antibody accessible to a solution flowing through each device. A lower absorbance is associated with a greater amount of accessible antibody. Error bars denote standard errors based on 8 independent measurements for each gel type.

Figure 2 shows yield and purity data for the capture of EPCs from whole blood using the hydrogel-coated microfluidic devices. In Figure 2a, gel type I, which has an inert antibody conjugated to it, shows negligible EPC adhesion as expected. Gel type II, which contains the anti-CD34 antibody, shows significantly higher EPC adhesion relative
to gel type I ($p < 0.005$), albeit with a high degree of scatter. The purity of capture achieved with gel type II is, however, relatively low (~23%; Figure 2b). The effect of adding the 4-arm PEG to the hydrogel structure is shown clearly by comparing gel types II and IV, whose synthesis protocol is otherwise identical. The branched amine termini of the 4-arm 10k MW PEG molecules provide an opportunity for a greater level of antibody conjugation,

![Figure 5. Illustration of structural differences in gel types. The left side of the figure is representative of mixing step 1 prior to the formation of amide bonds via](image)
EDC chemistry and the right side represents the product resulting from mixing step 2 (see also Table 1). The progressive improvement in EPC capture yield and purity from gel types III-VII is attributed to the two step mixing protocol where PEG and antibody are pre-mixed before the introduction of alginic acid and the coupling reagents EDC and sulfo-NHS. Pre-mixing allows optimal dispersion of antibody molecules among the PEG chains.

as reflected in the higher overall EPC adhesion (Figure 2a). The suppression of non-specific binding results in an increase in purity (Figure 2b; gel type IV). Interestingly, the use of 20k MW PEG (gel type III) resulted in significantly lower EPC capture yield relative to 10k MW PEG (gel type IV; $p < 0.005$) under the same synthesis conditions and purity levels were comparable.

In gel types V-VII, a two step protocol for combining reagents was followed. In gel type V, the conjugation of the antibody molecules to the 4-arm PEG is carried out first before introducing alginic acid. This formulation improved yield and purity of EPC capture relative to gel type IV. However, this protocol introduces the possibility of all four arms of the PEG molecules being occupied by antibodies, leaving no amine groups to bind to alginic acid. To address this risk, the two-step protocol was modified such that EDC and sulfo-NHS were added in the second step with alginic acid and the first step was restricted to the mixing together of PEG and antibody. When short times were provided for mixing and incubation for the first step (10 min and 15 min, respectively, for
gel type VI), the yield did not improve relative to gel type V, however purity was marginally higher. Higher mixing and incubation times were examined next (29 and 60 min, respectively, for gel type VII) in an effort to achieve greater mixing and entanglement of the PEG molecules with the antibody molecules. This formulation provided significantly higher yields and purity relative to gel types VI and VII ($p < 0.005$ and $p < 0.01$, respectively). For all gel types, the viability of captured and released cells, as measured by Trypan Blue exclusion, was approximately 90%.

### 3.5 Conclusion

The ability to selectively capture and then release cells within microfluidic channels requires a balance of physical forces and chemical reactions while simultaneously maintaining cell viability and function, particularly in the context of tissue engineering and regenerative medicine where cell isolation is a common initial step. Alginate hydrogels are particularly interesting for this application because of the ability to easily create them by physical crosslinking of alginic acid in the presence of divalent cations and their well-known ability to dissolve in the presence of chelators. Furthermore, these hydrogels are biocompatible and can be functionalized with cell-adhesive molecules. Alginate hydrogels are, however, highly prone to non-specific cell adhesion; for example, in a recent study by our group that examined the capture of fibroblasts by alginate hydrogels, there was appreciable cell adhesion to the non-functionalized hydrogel. When alginate hydrogels were functionalized with anti-CD34, a capture molecule for EPCs, and utilized for EPC capture from whole blood similar results were obtained in the form of high non-target cell adhesion (results not shown). The present study was motivated by these observations and the hypothesis that the
inclusion of PEG within the hydrogel structure would be a possible means to overcome this problem. Another shortcoming associated with functionalization of alginate hydrogels is the limited number of carboxylic acid groups available for carbodiimide-based conjugation. In the context of microfluidic capture devices, this limitation is exacerbated by the fact that flowing cells only “see” the adhesive capture molecules on the surface whereas conjugation protocols are easiest to carry out in the bulk prior to hydrogel formation within the microchannels.12

PEG is well known for its biological non-adhesiveness35-38 and the present study demonstrates how these properties can be harnessed while simultaneously increasing the capture molecule content of the hydrogel. These objectives are achieved by the use of 4-arm PEG molecules with primary amine terminations at the end of each arm. In an ideal case, one arm of each 4-arm PEG molecule would bind to a carboxylic acid group to the alginate hydrogel backbone, leaving up to three primary amine groups for antibody functionalization, provided the 4-arm PEG is sufficiently large to prevent steric hindrance between adjacent antibody molecules. This arrangement would, in principle, triple the antibody content of the hydrogel and provide protection against non-specific cell binding relative to non PEG-y-lated alginate hydrogels. However, such an idealized architecture is difficult to achieve synthetically if the same carbodiimide chemistry is to be utilized for both hydrogel-PEG and PEG-antibody binding. The present study examined how variations of a relatively simple synthetic protocol, made using probabilistic as well as chemical considerations, can overcome this constraint and provide effective capture and release of EPCs from whole blood. Protecting groups, such as fmoc,39 can be effectively employed to achieve some level of control over protein or antibody conjugation to
primary amine groups, however, here again it is difficult to ensure that only a certain number of amine groups are protected on each PEG molecule.

The need for simplicity in the hydrogel synthesis protocol arises from the intended application of these hydrogels in low-cost microfluidic cell separation systems for tissue engineering and clinical diagnostics. The present work, for example, provides the design basis for a microfluidic separator if EPCs from blood for subsequent use in vascular tissue engineering or cell-based regenerative repair of vascular tissue in vivo. Hence, there is significant motivation for keeping the number of synthesis steps and reagents to a minimum.

Figure 5 illustrates the evolution in synthetic protocols from gel types I through VII. The adhesive effect of the anti-CD34 antibody is evident by comparing gel types I and II (Figure 2a). Increased yield and purity are observed with the incorporation of 10k MW PEG (gel types II vs. IV), while the level of scatter remains relatively large. The large error bars in both instances reflect the random nature of bond formation between the alginate hydrogel and the amine group-containing antibody and PEG molecules. As shown pictorially in Figure 5a, it is likely that the 4-arm PEG molecules remain entangled in clusters, which would result in a patch like accumulation of these clusters within the hydrogel, with antibody molecules being bonded within the clusters as well as directly to alginic acid. Such irregular distribution of antibody molecules would give rise to relatively large variations in cell adhesion between hydrogel samples that are otherwise identical with respect to reactants and method of combination. Indeed, the alginic acid solutions obtained after the two synthetic steps for gel types II and IV contained small particles of PEG visible to the naked eye, corroborating the hypothesis of inadequate
mixing.

Each gel type is injected into a microfluidic device and subjected to identical flow and shear conditions. Slight variations in gel viscosity may impact the gel thickness within the device, however due to the geometry of the post array even a three-fold difference in gel thickness will impact the overall surface area by less than 6%. The differences in capture efficiency between gel types is far greater than 6% and it is therefore reasonable to argue that gel composition plays a much stronger role than gel thickness in the present context.

It is interesting to note that 20k MW PEG did not provide better capture and yield properties. Intuitively, one might expect the larger chains to provide a greater degree of steric freedom to antibody molecules bound to the amine termini and a greater resistance to non-specific binding (i.e. greater purity). However, the results in Figure 2 indicate lower yield and comparable purity relative to hydrogels containing 10k MW PEG (gel types III vs. IV). Both of these hydrogels have similar levels of antibody accessible to the BCA protein (Figure 4), indicating chemical structure similarity. Hence, the more likely explanation for this difference is a physical structure difference wherein antibody molecules are farther apart from each other with the 20k PEG- relative to the 10k PEG-containing hydrogel. The antibody molecules are far enough apart that their probability of encountering a flowing EPC and their ability to capture it are both lower with 20k PEG. On this basis, 10k PEG was utilized in all subsequent formulations.

With gel type V, the first step in the synthesis was the combination of PEG and antibody with the coupling agents EDC and sulfo-NHS prior to the addition of alginic acid in the second step. Relative to gel type IV, gel type V provides slightly greater EPC
capture but with a lower degree of scatter, indicating better mixing of the antibody molecules with the PEG. The accessible antibody content of gel type V is similar to that of gel type IV (Figure 4) providing credence to the postulate of better PEG-antibody mixing being the distinguishing factor. Better mixing also allows for more effective interspersing of PEG and antibody molecules on the hydrogel surface, which is consistent with the higher EPC purity obtained with gel type V relative to gel type IV. Fewer PEG particles were observed in the PEG- and antibody-functionalized alginic acid solution, consistent with this postulate.

The two-step synthesis protocol for gel types VI and VII built on the concept of better pre-mixing by providing time for antibody and PEG molecules to mix ‘undisturbed’ without the constraining presence of EDC and sulfo-NHS. Here, the longer mixing time provided alginic acid solutions that were visually clear, indicating good mixing, and the resulting coatings provided better EPC capture performance in terms of yield and purity relative to gel type V. The longer mixing and incubation times provided for gel type VII relative to gel type VI provided the best yield (~10^4 EPCs recovered) and purity (74%).

Figure 2a does not show a comparison of the EPC capture yields obtained with the hydrogels with the EPC content of whole blood. Flow cytometry measurements in our laboratory indicate a typical EPC count of 33 × 10^4 EPCs per 3 mL of whole blood for the samples used in this study. Hence the best performing gel type in this work only captures about a third of the total available EPC content. However, it important to note that this level of capture was achieved with only a single pass of the blood sample through the hydrogel-coated microfluidic devices. Series operation of multiple capture
devices or sample recycling would offer facile means to increase total yield. Stage-wise separation could be employed to increase the purity of captured EPCs beyond the 74% level achieved with gel type VII in a single pass. These considerations are outside the scope of the present work, where the focus was limited to the design and synthesis of the capture coating material.
4.0 Alternate Substrate Materials

4.1 Abstract

The use of poly(dimethyl siloxane) PDMS for microfluidic devices is ubiquitous in academic settings. However it is seldom used in commercial applications of microfluidic technology. Here I investigate the use of alginate hydrogels for cell capture on PDMS and a variety of materials more conventionally used in industry.

4.2 Introduction

One of the major obstacles in commercialization of microfluidic devices is the disparity between materials typically used for mass production in commercial processes, and the materials typically used in research laboratories. The limitations and challenges associated with using PDMS as a microfluidic substrate, including absorption of hydrophobic particles, its hydrophobicity, and non-ideal optical properties have been discussed by researchers for some time. Due to several factors, such as availability, robustness, and low-cost rapid prototyping, PDMS is appropriate and used extensively for research and development, even when other substrates may have better inherent properties for a particular application. This presents a significant obstacle to commercialization, since many protocols are developed specifically for PDMS, and those protocols may not function for other substrates that are more compatible with mass production. PDMS may be less favorable for mass production in some cases, since the cost of fabrication via soft lithography becomes high as compared to the cost of fabrication via injection molding as the number of parts increases. Thus, in order for a
microfluidics-based bioanalytical technique to gain widespread proliferation in clinical environments, it is critical that it be compatible with a variety of substrates.

Alginate hydrogels, for example, have been shown to be useful for rare cell capture when utilized in microfluidic devices fabricated from glass or poly(dimethylsiloxane) (PDMS). The efficacy of alginate-based cell-capture devices in other microfluidic substrates, such as polystyrene or Zeonor, however, is not well-characterized. The goal of this study is to evaluate the effectiveness of alginate hydrogels for rare cell capture applications in a variety of microfluidic substrate.

PDMS and glass microfluidic devices utilizing alginate hydrogels conjugated with anti-CD34 have been shown to be efficient in purifying Endothelial Progenitor Cells (EPCs)\(^2\). This study compares EPC cell capture efficiencies (using the same hydrogel-antibody capture mechanism) among devices fabricated from several different polymers including PDMS, and several commercially available ‘off the shelf’ chips fabricated from poly(methylmethacrylate) (PMMA), poly(carbonate) (PC), poly(styrene) (PS), and Zeonor. The ability to fabricate these chips from a variety of substrates is essential for more widespread adoption of microfluidics approaches to rapid cell purification in clinical settings.

### 4.3 Materials and Methods

Straight channel microfluidic chips were purchased from Microfluidic Chip Shop (Jena Germany) with the dimensions 2 mm X 18 mm X 0.15 mm. Chips were purchased in all available materials: PMMA, PC, PS and Zeonor. Additionally, chips with the same
dimensions were fabricated from PDMS and glass using standard soft lithography techniques.

Alginate gels were prepared using EDC/Sulfo-NHS chemistry as previously described. Gels were conjugated with a fluorescein isothiocyanate (FITC) IgG and injected into each device by hand. Bulk alginate was removed by flowing MES buffer at a rate of 5 µL/min for 20 min. to form a channel. The remaining alginate was gelled by introducing a 100 mM CaCl$_2$ solution at 5 µL/min for 20 min. Each channel was then photographed using a Nikon Eclipse microscope with a fluorescein (480±30/535±40 nm) excitation/emission filter to qualitatively verify the presence of alginate in each type of channel; results can be seen in Figure 5.

4.4 Results and Discussion

To verify the efficacy of each device for cell capture, alginate gels were conjugated with anti-CD34 capture antibody, a known surface marker for EPCs, injected into each device, and prepared as previously described. EPCs containing green fluorescent protein (GFP) were obtained from sheep and sorted using FACS. The cells were then prepared in a solution of serum-free cell growth medium at a concentration of 1 x 10$^6$ cells/mL. Cell solution was flowed through each device at a flow rate of 5 µL/min. for 40 min. The devices were then rinsed with MES buffer at the same flow rate for 10 minutes. Cells in each device were enumerated manually under a microscope, and micrographs were taken (Figures 6-7). Gels were then dissolved by introduction of the chelator ethylenediaminetetraacetic acid (EDTA) at 5 µL/min. for 20 min. Channels were rinsed by flowing MES buffer at 5 µL/min. for 20 min., and remaining cells were counted manually under a microscope. There was no difference (p > 0.05) in cell capture
performance between any of the five materials tested. A summary of cells in devices before and after introduction of EDTA is given in Figure 8.

Figure 6. Fluorescent images of alginate hydrogels conjugated with FITC formed in channels of (a) PC, (b) PMMA, (c) Zeonor, (d) PS, and (e) PDMS. The similarity in intensity demonstrates a similar level of gel adhesion to the substrate.
Figure 7. Microscope images of GFP cells adhered within microfluidic channels for (a) PC, (b) PMMA, (c) Zeonor, (d) PS, and (e) PDMS.
Figure 8. Manual count of cells adhered with alginate coated microfluidic channels before and after introduction of EDTA

4.5 Conclusion

These results show that an alginate hydrogel conjugated with capture antibodies is a versatile coating for cell capture applications, as it is functional in a variety of substrates. In fact, there is no statistically significant difference in the ability of the hydrogel to bind cells between any of the polymers tested. This suggests that an alginate-based mechanism will produce cell capture results similar to those seen in our previous work using PDMS across a variety of commercially available materials, including several that are more suited to mass production and subsequent widespread proliferation in clinical environments. Here we considered a very simple capture geometry in order to compare substrates; capture efficiency is expected to be greatly improved by introduction of more complex geometries, and/or by increasing the capture surface area, as was previously seen with PDMS.

The significance of this series of experiments is the demonstration of the ability to apply the PEG-alginate hydrogel coatings first described in Chapter 3 to substrates of any type. The development of such substrate-agnostic coatings and methods is essential for the translation of such technologies from the lab to commercial applications.
5.0 Capture Based on Multiple Markers

5.1 Abstract

Conventional cell separation against multiple markers typically requires the attachment of antibody tags, typically fluorescent or magnetic, to selected cell types in a heterogeneous suspension. This work describes how such separation can be accomplished in a series of microfluidic systems without the need for such tags. Two capture stages containing antibody-functionalized alginate hydrogels are utilized for the isolation of CD34+ and Flk1+ cells from untreated, whole human blood, respectively. The capture-release capability of these degradable coatings is harnessed by a mixing chamber and a simple valving system such that the suspension emerging from the first capture stage is prepared for the second capture stage for further enrichment. With this configuration we demonstrate the isolation of CD34+/Flk1+ endothelial progenitor cells from blood enabled by the depletion of CD34+/Flk1-hematopoietic stem cells population. This ability to achieve isolation of cells against multiple markers in an untagged separation method is of particular significance in applications involving cell implantation-based therapeutics including tissue engineering, and molecular analysis.

5.2 Introduction

The isolation of particular cell types from heterogeneous suspensions such as blood or digested tissue is an essential first step in many clinically-relevant protocols. Examples include from cell-based diagnostics, molecular analysis of cells via proteomics and genomics, and tissue engineering and cell-based therapeutics. For example, the ability to isolate endothelial progenitor cells (EPCs) from whole blood is desired for vascular tissue
engineering and cell-based therapeutic applications.\textsuperscript{1, 67, 68} EPCs are typically isolated from blood using a multi-cycle method of centrifugation and plating,\textsuperscript{1} which is a highly time-intensive process spanning days or weeks. Alternatively, these and other cells can be isolated at very high purity using the well-established techniques of fluorescence- and magnet-activated cell sorting (FACS and MACS, respectively). Both FACS and MACS require the attachment of antibody tags in the form of fluorescent dyes or magnetic beads, respectively, to one or more cell types in the sample. Such pre-processing tagging requires additional time and may be undesirable for cell-based therapeutic applications as well as in downstream molecular analysis. The approach of adhesion-based microfluidic cell separation aims to overcome this limitation via the use of immobilized antibodies and methods to release captured cells.\textsuperscript{69} A key challenge in this mode of separation is the ability to isolate target cells that do not have one unique surface marker that distinguishes them from the non-target cells in the sample. For instance progenitor cell markers expressed by EPCs such as CD34 are also expressed by other cell types present in blood, such as hematopoietic stem cells.\textsuperscript{70, 71} Furthermore, endothelial markers expressed by EPCs, such as Flk1 (also known as VEGFR-2 and KDR), are also expressed by mature endothelial cells.\textsuperscript{72, 73}

We recently described how alginate hydrogels co-functionalized with capture antibodies and poly(ethylene glycol) (PEG) are capable of accomplishing high purity capture of CD34\textsuperscript{+} cells from whole, untreated blood in microfluidic devices followed by efficient release.\textsuperscript{49} The release capability of these hydrogels arises from the ability to remove the divalent cations that physically-crosslink the alginate molecules to form the hydrogel using a chelator. This chapter describes the ability to extend this method to the
separation of cells expressing two different surface antigens, CD34 and Flk1. The scale-up from single to dual marker-based separation using the functionalized alginate hydrogels is not a trivial extension due to the need to achieve chelator based release in the first separation device without compromising the hydrogel coating in the second separation device and the need to neutralize the chelator molecules after cell release in the first separation stage.

5.3 Materials and Methods

The configuration of microfluidic devices for the dual marker separation described above is shown in Figure 9. Here, a sample is injected via a syringe pump into the first alginate-based capture stage (“Marker 1 isolation”/stage (i) in Figure 9). This stage is connected to stage (ii), which is a diverter valve chip. In its “closed” configuration, this valve lets the waste from stage (i) pass through to a collection tube. After all of the waste has gone through, the waste stream is closed using a clip. Next a solution of ethylene diamine tetraacetic acid (EDTA) is injected into stage (i) to release captured cells while simultaneously a 100 mM calcium chloride solution (in [2-(N-morpholino)ethanesulfonic acid] (MES) buffer) is injected into stage (ii). The purpose of the calcium chloride is to neutralize the EDTA in the cell suspension emerging from stage (i). To ensure mixing of the calcium chloride solution with this cell suspension, the combined output (which is in laminar flow) is sent into a mixing chamber (stage (iii)) containing herringbone features. The mixed solution then enters stage (iv) where the cells expressing receptors for the second capture molecule are captured. The final step in the separation process is the injection of an EDTA solution into the stage (i) inlet, which releases the captured cells from stage (ii). This solution is collected in a tube containing an excess of culture
medium in order to minimize any deleterious effect of the EDTA on the cells.

All of the chips shown in Figure 9 were fabricated using standard poly(dimethylsiloxane) (PDMS)-based soft lithography. Each of the capture devices is a post-array devices fabricated as described previously. Herringbone devices were fabricated with a single inlet and three outlets with an overall channel height of approximately 55 µm and herringbone features of approximately 20 µm in height.

Figure 9. Sequence of devices for adhesion-based microfluidic separation of cells against multiple surface markers. Following capture and release from device (i), cells expressing CD34 enter device (ii) where a calcium chloride solution is co-injected to neutralize the EDTA present in the cell suspension. Device (iii) mixes the calcium chloride solution and cell suspension. Device (iv) captures cells against the second marker, Flk1, which are eluted out by EDTA solution introduced at the inlet to device (i).

Two sets of dual-marker separation experiments were carried out. Preliminary experiments were carried out with ovine EPCs isolated as described by Kaushal et al.
Cell suspensions were prepared by removing cells from a culture flask with trypsin and diluting to a concentration of 100,000 cells per mL in serum-free EBM-2 medium (Lonza) and 100 µL were injected into the device array at a flow rate of 5 µL/min. The second set of experiments was performed with untreated whole human blood. Blood samples were drawn from healthy volunteers in heparin-coated Vacutainer collection tubes under a protocol approved by the Northeastern University Institutional Review Board. The blood samples were injected at a flow rate of 5 µL/min for 60 min.

Following the injection of homogeneous EPC suspensions or whole blood, the clip on the valve device (stage (ii) in Figure 9) was moved to send all effluent from stage (i) into the herringbone chip and the alginate coating within stage (i) was dissolved by introduction of the chelating agent, EDTA, at 10 µL/min for 10 min. The flow of EDTA into stage (i) was accompanied by the injection of calcium chloride solution into the second inlet of the valve chip at 5 µL/min. The EDTA and calcium chloride solution were then mixed in stage (iii). In order to maintain the same flow rate in the second capture device (stage (iv)), the flow exiting the herringbone mixer was divided into three streams and passed through three post array devices containing alginate conjugated with anti Flk1 capture antibody. Cells were released from stage (iv) by introduction of EDTA through the entire system at a flow rate of 30 µL/min (10 µL/min per stage (iv) device) for 10 min.

Characterization of the cells output from the multistage sequence was performed by conjugation of the recovered cells with anti-CD31-PE, anti-Flk1-APC, and anti-CD45-FITC (eBioscience) and enumeration using a Beckman Coulter Quanta SC flow cytometer. For comparison, separate experiments were carried out with both sample types
with only one capture device.

5.4 Results and Discussion

The output cell content from the single and dual capture configurations for cell capture from whole blood are shown in Figures 10 and 11. The CD34 receptor was targeted for the first stage of cell isolation based on the high expression level of this marker by the target population of EPCs. However, within the CD34+ population are CD31+ EPCs and CD45+ HSCs. The EPC content (CD31+/Flk1+) of the CD34 capture stage output is 37 ± 2% and the HSC content is 21 ± 1%. This suspension is processed by the valve chip and the herringbone mixer before flowing into the Flk1 capture stage. In the suspension recovered from the Flk1 capture stage, a major reduction in the number of CD45+ cells is readily apparent (Figures. 10 and 11). The EPC content of the Flk1 capture stage output is approximately 41 ± 4%. The output from this second capture stage also contains non-endothelial CD31+ cells (22 ± 11%), as evidenced by the greater number of CD31+ cells relative to the number of Flk1+ cells. Flk1 is expressed by EPCs as well as by more mature circulating endothelial cells and both of these populations are CD31+ in addition to being CD34+. It is known that CD31 is expressed by some subsets of leukocytes which are Flk1-. CD31 is also expressed by HSCs, which are also Flk1- and hence the total content of the CD31+/Flk1- leukocytes is 19 ± 11%.
Figure 10. Flow cytometry dot plots representing cells released from (a) the first stage and (b) the second stage. The y-axis represents CD45 expression and the x-axis CD31 expression. Blue dots represent red blood cells, red dots represent CD31+ and CD45+ positive cells (hematopoietic stem cells), green dots represent CD31+ and CD45- cells (EPCs).

The relatively large content of CD31+ leukocytes in the Flk1 capture stage output is surprising given their lack of expression of Flk1 as shown in Figure 11. While the antibody-functionalized alginate hydrogel coating in the Flk1 capture stage is quite
successful in suppressing the adhesion of CD45+ HSCs, the data indicate that is
susceptible to the adhesion of these CD31+ and Flk- leukocytes. For tissue engineering
applications, recovered cells will typically be placed in culture for expansion prior to
seeding on scaffolds; in this situation these leukocytes can be easily removed via a
medium change because they are non-adherent.
Figure 11. Performance of the multistage capture-release device system in dual-marker separation of EPCs from whole, untreated human blood. The target cells for these experiments were EPCs, which are CD34+ and Flk1+. The first capture stage is designed to selectively capture CD34+ cells. The second stage is designed to further enrich this population and remove CD34+ hematopoietic stem cells. The sample volume for each experiment was 300 μL of whole blood and numbers of cells in this figure are reported without any normalization. Cell counts were determined via flow cytometry. Error bars represent standard deviations based on 3 replicates.
Lastly, 35 ± 9% of the Flk1 capture stage output consists of red blood cells (RBCs). The presence of RBCs in this instance is attributable to physical trapping of these cells within the pillar arrays as opposed to extracellular matrix or surface receptor-mediated binding to device surfaces. As with the CD31+/Flk1- leukocytes above, these cells are non-adherent and can be removed when the recovered cells are placed in culture. Alternatively, mixing the recovered cell suspension with a lysis buffer is another means of eliminating these cells.

When the CD31+/Flk1- leukocytes and RBCs are excluded, the remaining cells consist of 92 ± 11% EPCs and 8 ± 1% HSCs. These values describe the composition of the adhered cells when the output of the Flk1 capture stage is placed in culture.

The efficiency of the capture devices utilized for both markers is around 60%. This value is consistent between the two sample types examined, namely homogeneous ovine EPC suspensions and whole blood. The efficiency of the second capture stage, by contrast, is higher, 75 ± 6% (obtained by comparing total numbers of cells recovered from each stage) and is attributable to the lower concentrations of cells entering the second capture stage relative to the starting sample that enters the first capture stage. In general, these levels of capture efficiency can likely be improved by optimizing the pillar array configurations to provide a greater likelihood of capture and by minimizing areas of non-homogeneous coating with the hydrogels. Still, the current work demonstrates reasonable levels of EPC recovery from the 300 µL volumes of the whole blood samples.

The significance of the separation platform described herein is the ability to carry
out an affinity separation of cells against two different surface markers from a complex sample without any tagging of target cells \textit{a priori}. The entire sequence of marker 1-based capture, release, mixing, and marker 2-based capture is accomplished simply by flowing a sequence of solutions in the following order (a) sample, (b) rinse solution (MES buffer), (c) EDTA solution which is mixed with calcium chloride solution in the mixing device and carries the recovered cells to the second capture stage, and finally (d) an EDTA solution to recover cells from the second capture stage and carry them into a container with excess culture medium to suppress any deleterious effects of the EDTA on the recovered cells. With the exception of the calcium chloride solution injected into the valve chip, all solutions are injected into the first capture chip, making the entire protocol easy to execute and automate.

\textbf{5.5 Conclusions}

Microfluidic devices containing alginate hydrogels functionalized with antibodies can be employed for sequential enrichment of a target cell population against two surface markers. Such enrichment can be accomplished by a serial array of microfluidic devices for capture and intermediate stage devices to neutralize the chelator utilized to release cells from the first stage. The strength of the method lies in the relative simplicity of the layout and the reasonable levels of cell recovery from a complex sample.
6.0 Comparison of Adhesion-Based Microfluidic Separation of CD34+ Cells with MACS

6.1 Abstract

Magnet actuated cell sorting (MACS) is a widely used technique for enriching cells. Here I compare MACS with alginate coated microfluidic devices for cell enrichment. The data will show that in all relevant metrics microfluidic cell separation performs at least as well as MACS.

6.2 Introduction

The isolation of cells from a variety of heterogeneous and complex suspensions such as blood or digested tissue is necessary for a myriad of applications including tissue engineering, diagnostics and cellular biology. Currently, the gold standard for such separations is magnetic actuated cell sorting (MACS). However effective, MACS presents a few inherent drawbacks such as the inability to process samples with a larger number of contaminants for example, whole blood and the inability to remove the magnetic tags from the isolated cell populations.\textsuperscript{82} Recently, it has been shown that microfluidic devices can be used to accomplish this separation.\textsuperscript{48, 49} In this study the new microfluidic isolation technique is compared directly to commercially available MACS columns in the isolation of endothelial progenitor cells (EPCs) from human blood.

6.3 Materials and Methods

Using anti CD34 as a capture moiety, cells were isolated from human blood using both MACS columns and microfluidic post arrays. Microfluidics devices were injected with whole blood as previously described.\textsuperscript{49} Briefly, blood was flowed through
microfluidic chips at a flow rate of 5 µL/min and subsequently eluted using a 50 mM solution of ethylene diamine tetraacetic acid (EDTA) at a flow rate of 10 µL/min.

Blood was collected from healthy volunteers by routine venipuncture into four CPTTM Tubes with heparin (BD Biosciences, Franklin Lakes, and NJ). After centrifugation at room temperature in a swinging bucket rotor for 20 min at 1500 RCF the mononuclear cells were collected and diluted with phosphate buffered saline supplemented with 2 mM EDTA (PBSE). The cells were centrifuged for 15 min at 300 RCF and the cell pellet washed; this procedure was repeated once. Each 3.3 x 10^7 cells peripheral blood mononuclear cells (PBMCs) was resuspended in 100 µL of PBSE to which 33 µL of FcR blocking reagent (Miltenyi Biotec Inc., Auburn, CA) and 33 µL of magnetic microbeads conjugated with an anti-CD34 antibody was added. After incubation for 30 min at 4°C, the cells were diluted in 10 x volume of PBSE supplemented with 0.1% bovine serum albumin. The CD34+ cells were positively selected using the automated magnetic selection autoMACSTM (Miltenyi Biotec Inc., Auburn, CA) following the manufacturer's instructions. The selected cells were confirmed to be CD34+ cells by co-staining with PE conjugated anti-CD34 (Miltenyi Biotec Inc., Auburn, CA.)

We were able to detect the CD34+ cells migration using a modified Boyden chamber (Neuroprobe, MD) with separation polycarbonate membrane, 8 µm pores (Neuro Probe, Gaithersburg, MD) coated with 10% bovine collagen. Approximately 10,000 CD34+ cells were labeled with CellVue Burgundy (Molecular Targeting Technologies, West Chester, PA). Then the cells were loaded into upper part of the chamber and SDF1 was in the lower part. After 4 h at 5% CO2 at 37°C, the percentage of cells that migrated
was determined by collecting media in the lower chamber and determining relative fluorescence using Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) at excitation of 683 nm and an emission of 707 nm. Percentage increase in migration was calculated as maximum increase in migration in response to SDF as compared to baseline (no SDF).

### 6.4 RESULTS AND DISCUSSION

In whole blood there are primarily three markers that can be used to identify a cell as an EPC. CD34, a progenitor cell marker, CD133, another progenitor cell marker that down regulates more rapidly and is therefore less promiscuous, and CD45, which is a blood cell marker present on most white blood cells. There are generally two cell types that are relevant when discussing EPC isolation. Hematopoietic stem cells which are positive for CD34, CD133 and CD45 and EPCs which are also positive for CD34 and CD133 but do not express CD45. Therefore it is of interest to compare purities and cell counts for those groups. Figure 12a illustrates the purity for early and late stage EPCs as well as the depletion of the CD45+ white blood cells. Purity is comparable between the two separation techniques while figure 12b demonstrates the difference in the total number of cells captured. Microfluidic isolation has a higher yield in terms of number of cells captured, this is due partially to the fact that the microfluidic devices process whole blood directly while the MACS columns require preprocessing steps in the form of centrifugation which can result in the loss of cells.
Figure 12. Purity (A) and yield (B) for different marker combinations.

Microfluidics provides significantly higher yield (number of cells recovered) for CD34+/CD45- and CD45- fraction with comparable purity relative to MACS. Purity and yield are statistically similar and relatively much smaller for the CD34+/CD133+/CD45+ fraction. Error bars represent standard errors based on 9 samples for microfluidic and 2 samples for MACS.

Microfluidic chips presented a significant depletion of CD45+ blood cells when compared to MACS as shown in Figure 13 A-C. As EPCs mature from progenitor cells to subsequent lineages the expression of CD133 decreases precipitously, the expression of CD34 also decreases but with some time delay relative to CD133. It is of interest to differentiate between cells with high CD133 expression the so-called early stage EPCs and the lower CD133 but still high CD34 populations or the later stage EPCs. The microfluidic chips give greater access to the early stage EPCs than do the MACS columns as demonstrated by the difference between the plots shown in Figure 13e and
Figure 13. (A-C) Flow cytometry based assessment of relative CD45+ content in whole blood and isolates from CD34 positive selection via microfluidics and MACS. MACS isolation using CD34 positive selection beads results in appreciable CD45+ content, as shown in (C). Microfluidics, by contrast, as much lower CD45+ content. This is likely a consequence of EPC adhesion anti-CD34 surfaces within the microfluidic chips being stronger in the context of flowing fluid relative to CD45+ hematopoietic stem cells. Photograph of microfluidic chip (D). Relative content of CD133+ and CD45+ cells in microfluidic and MACS isolates. Microfluidics provides better access to early stage CD34+ and CD133+ progenitors (P4 and P7).
6.5 CONCLUSION

The comparison of microfluidic isolation and MACS shows that similar purities are obtained by both methods, both methods provide cells with minimal activation, however due to the need for preprocessing steps for MACS columns the overall yield of cells can be higher in microfluidic devices. Additionally microfluidics provides access to high numbers of both early and late stage EPCs. This has the potential to improve isolation used for many downstream processes and may open the door to new avenues of study for EPCs as well as other cell types.
7.0 Comparison of Adhesion-Based Microfluidic Separation of CD34+ Cells with Conventional Preplating Technique

7.1 Abstract

A subset of EPCs that have the ability to form colonies in culture are referred to as endothelial colony forming cells (ECFCs). These cells are of great interest to scientists, however due to their rarity they are difficult to access. This study compares conventional isolation methods with a novel microfluidic based isolation protocol. Microfluidic cell isolation was compared directly to traditional centrifugation and preplating methods. Cells isolated by both techniques were compared phenotypically as well as via in vivo and in vitro assays. In all cases the microfluidic cells performed as expected for the cell type and microfluidics seems to work at least as well as conventional techniques.

7.2 Introduction

A subset of EPCs is the endothelial colony-forming cell (ECFC). These are circulating endothelial cells, which have the ability to form colonies in culture. These cells can expand in culture to a great extent. As a result there are of great interest to clinicians and researchers. These cells are useful for the endothelialization of vascular grafts as well as cellular based therapies. When implanted into mouse models these cells have shown to form long lasting vascular structures with the ability to form anastomes, that is interact with the hosts native vasculature. These characteristics make ECFCs well suited for vascular tissue engineering applications and autologous cellular based therapies.

Despite the great potential of these cells for basic science and therapeutic
applications, there are a few drawbacks. These cells are rare in peripheral blood making their isolation challenging. They have been reported to exist in concentrations below 1 per 10 million mononuclear cells in peripheral blood in healthy adults. In fact many studies have reported an apparent absence of ECFCs in some population of healthy adults. Therefore it is of interest to direct development into more reliable and robust methods for isolating these cells from adults.

The current gold standard for isolating ECFCs is plating of the mononuclear fraction of blood and allowing them to grow. Then visually identifying the ECFCs for manual isolation and subsequent plating. Due to the low frequency of these cells in blood the success of this approach requires large blood volume or many trials to obtain sufficient colony forming units. This can be exceedingly difficult in certain clinical situations where access to sufficient blood volumes becomes problematic. Additionally due to the rarity of these cells there exists a lack of distinctive markers for use in other methods such as FACS or MACS techniques. Due to the processing and preprocessing steps these techniques may have deleterious effects on the actual recovery of cells. A method that can isolate these cells from a small volume of unprocessed blood would be of great interest to the scientific community.

Microfluidic cell purification systems may be the platform to achieve these goals. Microfluidic systems have been shown to isolate rare cells with high purities in an efficient and rapid manner. As previously described in this work, the degradable hydrogel coating may be ideally suited for this purification from small volumes of unprocessed blood. Here, we reason that this microfluidic approach can be instrumental in isolating rare circulating ECFCs for growth in culture. We have examined the
feasibility of capturing ECFCs from whole peripheral blood in four adult volunteers and conducted preclinical experiments to validate the phenotypical and functional properties of the captured ECFCs, including the ability to form microvascular networks in vivo.

7.3 MATERIAL AND METHODS

7.3.1 Microfluidic device design and fabrication

The design and fabrication of the micropost array microfluidic devices followed previously described soft lithography techniques. First, a negative master was fabricated and assembled at the George J. Kostas Nanoscale Technology and Manufacturing Research Center at Northeastern University using conventional photolithography techniques. Briefly, a silicon wafer was coated with SU 8-50 photoresist to a thickness of approximately 50 µm. With the transparency overlaid, the wafer was exposed to 365 nm, 11 mW/cm² UV light from a Q2001 mask aligner (Quintel Co). The unexposed photoresist was then removed using SU 8 developer. Feature height was verified using a Dektak surface profiler (Veeco Instruments). Post-array devices consisting of 100-µm diameter posts with a gap, edge-to-edge distance of 50 µm were fabricated. Posts were arranged in a hexagonal pattern, where three adjacent posts form an equilateral triangle pattern. The overall dimension of the device was 5 x 7 x 0.05 mm. To form the polymeric chambers, poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning) elastomer was mixed (10:1 ratio) and poured onto a negative master, degassed, and allowed to cure overnight. PDMS replicas were then removed; inlet and outlet holes were punched with a 19-G blunt-nosed needle. Replicas and glass microscope slides (25 x 75 x 1 mm³) were then exposed to oxygen plasma and placed in contact to bond irreversibly. Micropost
surface modification was carried out as previously described.\textsuperscript{49}

Briefly, Poly(ethylene glycol) (PEG) and alginate (Sigma) were mixed in an equal molar ratio, combined with EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] and sulfo-NHS (N-hydroxysuccinimide) (Fisher Scientific). The mixture was then injected by hand into the microfluidic devices and allowed to incubate for 60 minutes. The bulk gel was then removed from the device by flowing 2-(4-morpholino)ethanesulfonic acid (MES) buffer at 10 µL/min. The remaining gel was solidified by injection of 100 mM calcium chloride solution at 10 µL/min. Mouse anti-human CD34 antibody (Santa Cruz Biotechnology) at a concentration of 0.01 mg/mL was combined with EDC and sulfo-NHS, injected by hand, and allowed to incubate for 30 minutes.

\textbf{7.3.2 Blood collection}

Adult peripheral blood (12 mL) was collected from volunteer donors by venipuncture in accordance with an Institutional Review Board-approved protocol and with informed consent according to the Declaration of Helsinki. Blood samples were collected in heparinized tubes (Becton Dickinson). Each sample was split into two (6 mL each); one half was processed following the standard ECFC isolation method and the other half using our microfluidic device.

\textbf{7.3.3 Standard ECFC isolation method}

Blood (6 mL) was diluted 1:1 with isolation buffer (PBS, 0.6% ACD-A, 0.5% BSA) in a centrifuge tube and was overlaid onto an equivalent volume of Ficoll-Paque
Plus (Amersham Pharmacia). Cells were centrifuged for 15 min at room temperature at 2,700 rpm. Mononuclear cells (MNCs) were gently collected, washed two times with isolation buffer, diluted 1:1 with ammonium chloride solution (StemCell Technologies), and incubated for 5 min on ice for erythrocyte lysis. MNCs were then washed two times with isolation buffer and cultured on 1% gelatin-coated plates using ECFC-medium: EGM-2 (except for hydrocortisone; Lonza) supplemented with 20% FBS, 1X glutamine–penicillin–streptomycin (GPS; Invitrogen). Unbound cells were removed at 48 h and the bound fraction maintained in ECFC-medium, with media being replenished every 2-3 days.

7.3.4 Microfluidic ECFC isolation method

Whole blood (6 mL) was directly flowed through 20 parallel microfluidic devices with functionalized posts at 5 µL/min/device using a Harvard Apparatus PHD2000 syringe pump (Harvard Apparatus). The blood was then rinsed from the device using MES buffer at a flow rate of 10 µL/min to a total volume of 100 µL (10 min) followed by an injection of a 50 mM solution of ethylene diamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) (Fisher Scientific) to elute cells. The eluted cell fraction was then cultured on 1% gelatin-coated plates using ECFC-medium. Unbound cells were removed at 48 h and the bound fraction maintained in ECFC-medium, with media being replenished every 2-3 days.

7.3.5 ECFC culture

ECFC colonies were identified as well-circumscribed monolayers of >50 cells with typical cobblestone morphology. ECFC colonies were enumerated on day 28 by
visual inspection using an inverted microscope. At confluence, ECFCs were purified using CD31-coated beads (Dynal Biotech) as previously described [1]. CD31-selected ECFCs were routinely subcultured on 1% gelatin-coated plates using ECFC-medium. ECFCs are hereafter referred to as std-ECFCs (standard isolation method) and mf-ECFCs (microfluidic isolation method). Both std-ECFCs and mf-ECFCs were used between passages 4-8 in all our studies.

7.3.6 Flow Cytometry

Flow cytometry analyses were carried out by labeling cells with either phycoerythrin (PE)-conjugated mouse anti-human CD31 (1:100; Ancell), PE-conjugated mouse anti-human CD90, fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD45, FITC-mouse IgG1, or PE-mouse IgG1 antibodies (1:100; BD Biosciences). Antibody labeling was carried out for 20 min on ice followed by 3 washes with 1% BSA, 0.2 mM EDTA in PBS and resuspension in 1% paraformaldehyde in PBS. Samples were analyzed with a Guava easyCyte 6HT/2L flow cytometer (Millipore Corporation) and FlowJo software (Tree Star Inc.).

7.3.7 Immunofluorescence

Immunofluorescence was carried out using mouse anti-human CD31 (1:200; DakoCytomation), mouse anti-human vWF (1:200; DakoCytomation), and goat anti-human VEcadherin (1:200; Santa Cruz Biotechnology) antibodies, followed by FITC-conjugated secondary antibodies (1:200; Vector Laboratories) and Vectashield mounting medium with DAPI (Vector Laboratories).
7.3.8 Proliferation assay

ECFCs were seeded in triplicate onto fibronectin-coated 24-well plates at 5x10^3 cell/cm^2 using basal medium (EBM-2, 5% FBS). Plating efficiency was determined at 24 h. Cells were then treated for 48 h using basal medium in the presence or absence of either 10 ng/mL VEGF-A or 1 ng/mL FGF-2 (R&D Systems). Cells were quantified under a fluorescent microscope after DAPI staining using ImageJ analysis software (National Institutes of Health); results were normalized to cell number obtained in basal medium.

7.3.9 Scratch assay

The scratch assay was performed in confluent cultures of ECFCs plated on 6-well plates. Scratch wounds were generated across each well using a pipette tip. Cells were then treated for 24 h using basal medium in the presence or absence of 10 ng/mL VEGF-A or 1 ng/mL FGF-2. Scratch size was measured after 24 h under a phase contrast microscope.

7.3.10 Tube formation assay

ECFCs were seeded on Matrigel-coated plates at a density of 2x10^4 cell/cm^2 and incubated for 24 h in ECFC-medium. The total length of ECFC-lined cords were measured using ImageJ software.

7.3.11 Up-regulation of leukocyte adhesion molecules

ECFC monolayers were challenged with or without 10 ng/ml of tumor necrosis
factor-α (TNF-α; R&D Systems) for 5 h. Afterwards, the leukemia cell line HL-60 (2x10^6 cells) was added and incubated at 4 °C on a rocking platform for 45 min. Bound leukocytes were visualized using a phase contrast microscope and quantified with ImageJ software. Additionally, leukocyte adhesion molecules were analyzed by flow cytometry using PE-conjugated antibodies against human E-selectin and ICAM-1 (1:100; BD Biosciences).

7.3.12 In vivo vasculogenic assay

Six-week-old athymic nu/nu mice were purchased from Massachusetts General Hospital (Boston, MA). Mice were housed in compliance with Boston Children’s Hospital guidelines, and all animal-related protocols were approved by the Institutional Animal Care and Use Committee. Vasculogenesis was evaluated in vivo using our xenograft model as previously described. Briefly, ECFCs and human bone marrow-derived mesenchymal stem cells (MSCs) (two million total; 2:3 ECFC/MSC ratio) were resuspended in 200 µl of Matrigel (BD Biosciences) and the mixture was subcutaneously injected. All experiments were carried out in 4 mice.

7.3.13 Histology and immunohistochemistry

Implants were removed from euthanized mice, fixed in 10% buffered formalin overnight, embedded in paraffin, and sectioned (7-µm-thick). Hematoxylin and eosin (H&E) stained sections were examined for the presence of blood vessels containing red blood cells. Microvessel density (vessels/mm²) was reported as the average number of erythrocyte-filled vessels in sections from the middle of the implants. For
immunohistochemistry, sections were deparaffinized, and antigen retrieval was carried out by heating the sections in Tris-EDTA buffer (10 mM Tris-Base, 2 mM EDTA, 0.05% Tween-20, pH 9.0). Sections were blocked for 30 min in 5-10% blocking serum and incubated with primary antibodies for 1h at room temperature. The following primary antibodies were used: mouse anti-human CD31 (1:50; DakoCytomation, M0823 Clone JC70A), mouse anti-human α-SMA (1:200; Sigma-Aldrich, A2547 Clone 1A4), and mouse IgG (1:50; DakoCytomation). Horseradish peroxidase (HRP)-conjugated mouse secondary antibody (1:200; Vector Laboratories) and 3,3’-diaminobenzidine (DAB) were used for detection of human CD31, followed by hematoxylin counterstaining and Permount mounting. Fluorescent staining were performed using rhodamine-conjugated UEA-1 (20 µg/mL) and FITC-conjugated secondary antibodies (1:200; Vector Laboratories) followed by DAPI counterstaining.

7.3.14 Microscopy

Images were taken using an Axio Observer Z1 inverted microscope and AxioVision Rel. 4.8 software (Carl Zeiss). Phase microscopy images were taken with an AxioCam MRm camera and 5x/0.16 or 10x/0.3 objective lens. Fluorescent images were taken with an ApoTome.2 Optical sectioning system (Carl Zeiss) and 20x/0.8 or 40x/1.4 oil objective lens. Non-fluorescent images were taken with an AxioCam MRc5 camera using a 40x/1.4 objective oil lens.

7.3.15 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Means were compared using unpaired Student’s t tests. Comparisons between multiple groups were
performed by ANOVA followed by bonferroni post-test analysis. All statistical analyses were performed using GraphPad Prism v.5 software (GraphPad Software Inc). P<0.05 was considered statistically significant.

7.4 Results And Discussion

Cells isolated via microfluidics were evaluated for function and phenotype. As can be seen in Figure 14, cells isolated via microfluidics are capable of proliferation in culture. Additionally Microfluidics performed at least as well as conventional with 3 of 4 samples producing growth colonies. These cells express CD31 indicating that they are endothelial in nature while not expressing CD45 showing that they are not blood cells but vascular in nature.
Cells were compared to conventionally isolated cells in both *in vivo* and *in vitro* studies. Microfluidic purified cells performed as expected in both types of assays and shown in Figures 15 and 16. Cells respond to stimulation by TNF-α as expected in ICAM-1 and E-selectin upregulation. Additionally TNF-α induces adhesion in the presence on leukocytes as shown in Figure 15A-B. Proliferation of cells are similar to standard isolated cells as shown in Figure 15C. The scratch test assay demonstrated migratory abilities of microfluidic isolated cells in the presence and absence of VEGF.
(see Figure 15D). Additionally the live dead assay indicated the majority of cells are viable after purification as can be seen in Figure 15E. There is no ostensible or statistical difference between the two cell populations indicating that the cells are agnostic to the separation type if there are colony forming units present in the culture after purification.

Microfluidic isolated cells were implanted into a nude mouse host. These cells behaved as expected in all assays performed. Cells were capable of forming vascular bundles in the host as can be seen in Figure 16A-C. Figure 16D demonstrates that the vascular bundles are of human origin by staining with human CD31. This indicates that the cell growth was derived from the implanted cells and is not growing from the host’s native vasculature. Cells express smooth muscle actin (SMA) as seen in Figure 16E-F. This is seen in cells surrounding the lumen and indicate good perivascular coverage indicating stability. In summary the results of Figures 15 and 16 demonstrate the microfluidic-isolated cells perform in a similar fashion to those derived via the standard method.
Figure 15. Standard *in vitro* assays comparing microfluidics and cells derived via conventional centrifugation and preplating. A) ICAM-1 and E-selectin are upregulated when cells are stimulated by TNF-α. B) Stimulation by TNF-α promotes the adhesion of leukocytes to EPC layer in culture. C) Proliferative abilities of cells isolated by microfluidics and standard method. D) Scratch assay to demonstrate migration in the presence and absence of VEGF. E) Live dead assay of microfluidic isolated cells and comparison to standard method.
7.5 CONCLUSION

Microfluidic isolation of ECFCs has shown to produce significant numbers of
colony forming units from small blood volumes (3-6 mL). These cells when harvested grow in a manner that is similar to those isolated by conventional methods. In both in vivo and in vitro assays the cells performed as expected and consistently with previous work described in literature. This indicates that microfluidic isolation can be a viable option for the preparation of ECFCs for use in autologous cell therapies, vascular tissue engineering, and basic science research.
8.0 Optimization of Culture Protocol for CD34+ Cells Separated via Microfluidics from Whole Blood

8.1 Abstract

Three different media types were compared for growth of released EPCs, EBM-2 with EGM-2, EBM-2 with EGM-2 MV, and M199. In each case the cell growth was monitored for 15 days while the media was changed every three days. Cell growth is similar for all three types for the first week after which M199 seems to have the best results in terms of proliferation. Cells can be transported most successfully if the transport takes place prior to the initial cell adhesion.

8.2 Introduction

The ability of purified cells to proliferate in culture is of critical importance to their usefulness in both cellular based therapies and tissue engineering applications. Cells that can be expanded to significant numbers are useful for autologous cell implantation. Additionally large numbers of cells may be required to seed scaffolds for tissue growth. Due to the rarity of EPCs it is not feasible to purify sufficient numbers to meet the needs of these applications. Therefore purifying a smaller number of cells and growing them in culture is of interest to the end uses of this project. In an attempt to optimize this growth, several media types were compared EBM-2 with EGM-2 bullet kit, EBM-2 with EGM-2 Micro Vascular (EGM-2MV) bullet kit, and M199. Cells were isolated divided and added to each medium. The motivation for this study was to find the optimal conditions for isolating cells and subsequently transporting them to a location where they would be used. This initially proved difficult. The motion of the media during transport tends to shear the cells from the surface in such a way that they are loath to reattach.
8.3 Materials Methods

Cells were captured and released in ten microfluidic devices as previously described. Cells captured from blood cells were released into one of three culture mediums and placed in an incubator. Growth medium was replaced after 48 hours and then every 72 hours following. Microfluidic isolated cells were compared with a control of FACS isolated ovine EPCs. Growth was then monitored visually using a microscope over a two-week period. Cells were counted on five occasions during those two weeks. For this study the counting consisted of counting all cells in a 10X field view. Three random positions were counted and averaged.
Figure 17. a) Released cells at day 2 (b) Released cells after 10 days. The cells are adhering to the substrate and dividing to demonstrate the ability of released cells to proliferate. $5 \times 10^5$ GFP labeled sheep EPCs were spiked into 3 ml of whole sheep blood and processed through 5 devices.

8.4 Results and Discussion

The released cells grew in a manner that is visually indiscernible from those cells isolated by FACS as can be seen in Figure 17. The number of cells is initially very similar and remains so for the first eight days as can be seen in Figure 18. However after day eight a divergence begins to occur. The cells grown in the M199 media seem to continue to proliferate more rapidly than the EGM2 mediums. This initially gave an indication that
culturing the cells for a short time in M199 would render the cells suitable for transport. Later trials of this theory proved fruitless. Additional work demonstrated the ability to transport cells immediately after purification before adhesion had taken place. This allowed the cells to move without damaging their ability to adhere to the substrate.
Figure 18. Growth data for three different types of media EBM-2 with EGM-2, EBM-2 with EGM-2 MV, and M199. Cell counts represent number of cells 10X field view in three random locations. Error bars are standard deviation.
8.5 Conclusion

EPCs can grow in several types of media. It appears that there is little difference between the EGM-2 and EGM-2 MV bullet kits when it comes to EPC proliferation. M199 caused cells to proliferate more rapidly for a more sustained period of time. Cells can be transported more successfully if done so immediately after isolation prior to initial cell adhesion. Other factors that are of relevance but not measured in this study are the impact of the different cell types on cell differentiation. From this study it is not clear that cell differentiation is the same in all cases. This can be a subject of future work.
9.0 Overall Conclusion

In this work it was demonstrated that using an alginate PEG hydrogel functionalized with the capture molecule CD34 it is possible to isolate EPCs from whole blood in a microfluidic device. This can be expanded to isolate cells based on multiple markers in any desired combination. This coating of hydrogel is agnostic to microfluidic material substrates. The coating was successfully applied to many materials including polystyrene, polycarbonate, and glass. The cells isolated by this method retain their viability and are able to proliferate in culture. The cells perform as expected and at least as well as cells isolated by centrifugation and pre-plating. Cell purities are comparable to those achieved by MACS but purified cell numbers are much higher. This indicates that use of this method as a means to purify cells could be of use to a variety of laboratory settings and applications.
10.0 References


