A Novel Vascular Graft Diagnostic and
Reversible Aptamers for the Purification of Therapeutic Cells

by

Michael Douglas Nichols

Department of Biomedical Engineering
Duke University

Date: __________________________
Approved:

___________________________
William M. Reichert, Supervisor

___________________________
Bruce A. Sullenger

___________________________
Rebekah R. White

___________________________
Charles A. Gersbach

___________________________
Bruce M. Klitzman

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Biomedical Engineering
in the Graduate School of Duke University

2017
ABSTRACT

A Novel Vascular Graft Diagnostic and
Reversible Aptamers for the Purification of Therapeutic Cells

by

Michael Douglas Nichols

Department of Biomedical Engineering
Duke University

Date: _______________________
Approved:

________________________________
William M. Reichert, Supervisor

________________________________
Bruce A. Sullenger

________________________________
Rebekah R. White

________________________________
Charles A. Gersbach

________________________________
Bruce M. Klitzman

An abstract of a dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Biomedical Engineering
in the Graduate School of Duke University

2017
Abstract

Creation of novel tools for biomedical applications is critical for the improvement of patient diagnostics and therapeutics. Two particularly important needs lie in (1) improved in vitro testing and increased performance of prosthetic vascular grafts and (2) purification methods for cells that do not compromise their utility. Progress in these areas is urgently needed and would facilitate the availability of higher quality devices and treatments that raise the quality of patient care. This work focused on developing new approaches toward that goal.

A tremendous and immediate need exists for high-performance small-diameter synthetic vascular grafts, as a fifth of the 500,000 annual coronary artery bypass grafting (CABG) patients lack suitable autologous vessels for revascularization. This problem has driven intense research and development of increasingly diverse prosthetics that could be viable alternatives in the years to come. Evaluating these designs in vitro offers high-throughput, low-cost screening for promising graft technologies ahead of more stringent vetting in vivo.

Offering a fresh take on assessing vascular graft thrombogenicity in vitro, the buildup of pressure upstream to a clot was used as a metric to quantify the physical interaction between the graft lumen and a maturing thrombus. A closed tubing system was devised and continuously monitored as clotting solutions of fibrin glue, platelet-
rich plasma or whole blood were cured to varying maturities and then purged from small-diameter ePTFE grafts or Tygon graft mimics. This approach provided insight into how blood flow resistance is influenced by a number of clinically relevant factors, such as the level of vessel occlusion and the physical nature of the resident coagulum.

Endothelialization of synthetic vascular grafts yields viable alternatives to native vessels and can be accomplished non-invasively using late-outgrowth endothelial progenitor cells (LO-EPCs) isolated from peripheral blood. However, the time required to amass sufficient cells to prepare a graft with current methods is risky for waiting CABG patients. An ambitious approach conceived to significantly decrease this wait period involved developing affinity ligands selective for LO-EPCs that would enable their capture directly from the circulation to facilitate rapid amassment. An in vitro directed evolution strategy to generate aptamers, the nucleic acid analogs of antibodies, that specifically bind these cells was carried out with initially promising but ultimately unsuccessful results. While the particular strategy executed here did not prevail, the high value and impact LO-EPC aptamers would deliver merit revisiting this work with a revised strategy such as the one proposed in this document.

Purification of autologous and allogenic cells is essential for their use in a variety of therapeutic and basic research applications in addition to augmenting graft performance. However, the antibody stains conventionally used to selectively purify
cells are permanent and their continued presence can elicit an immune response in vivo and compromise native cell behavior. To avoid these issues, a cell purification strategy was crafted utilizing aptamers and matched oligonucleotide antidotes that enabled reversible cell staining. The reversible stains were robust enough for cell purification via fluorescence-activated cell sorting (FACS) yet subsequently able to be removed with gentle heat treatment and antidote. Importantly, cell function that was compromised without antidote was rescued to match the native behavior of non-stained cells following purification and antidote treatment.
Dedication

This work is dedicated to Wendy, Olive and yes - Louie too.
## Contents

Abstract ............................................................................................................................................. iv

List of Tables ........................................................................................................................................ xv

List of Figures ....................................................................................................................................... xvi

Acknowledgements .............................................................................................................................. xxvii

1. Introduction ....................................................................................................................................... 1

1.1 Synthetic vascular grafts .................................................................................................................. 1

1.1.1 Clinical significance of high-performance synthetic vascular grafts ........................................ 1

1.1.2 Vascular grafts failure modes ...................................................................................................... 3

1.1.3 Graft material treatments and coatings ....................................................................................... 4

1.1.4 Endothelialization ....................................................................................................................... 7

1.1.4.1 Endothelial cell (EC) attachment to graft surfaces ............................................................... 9

1.1.4.2 EC sources and types ............................................................................................................. 12

1.1.5 Vascular graft assessment modalities ......................................................................................... 13

1.1.5.1 In vivo .................................................................................................................................... 14

1.1.5.2 Ex vivo ................................................................................................................................... 15

1.1.5.3 In vitro ................................................................................................................................... 16

1.2 Endothelial progenitor cells (EPCs) ............................................................................................... 17

1.2.1 Contemporary knowledge of EPC subclasses ............................................................................ 18

1.2.2 Sourcing of late-outgrowth EPCs (LO-EPCs) ............................................................................ 20

1.2.3 Clinical significance of LO-EPCs ............................................................................................... 21
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.3.1 Therapeutic significance</td>
<td>21</td>
</tr>
<tr>
<td>1.2.3.2 Diagnostic significance</td>
<td>22</td>
</tr>
<tr>
<td>1.2.4 Poorly defined biomarkers</td>
<td>25</td>
</tr>
<tr>
<td>1.2.5 Challenges of contemporary LO-EPC isolation strategies</td>
<td>27</td>
</tr>
<tr>
<td>1.3 Aptamers for high-fidelity cell discrimination and purification</td>
<td>28</td>
</tr>
<tr>
<td>1.3.1 Aptamers</td>
<td>28</td>
</tr>
<tr>
<td>1.3.2 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)</td>
<td>29</td>
</tr>
<tr>
<td>1.3.2.1 Subtractive whole-cell SELEX and biomarker discovery</td>
<td>30</td>
</tr>
<tr>
<td>1.3.2.2 Efficient aptamer candidate screening via High-Throughput Sequencing (HTS)</td>
<td>33</td>
</tr>
<tr>
<td>1.3.3 Comparison to peptides</td>
<td>34</td>
</tr>
<tr>
<td>1.3.4 Antidote control of aptamer activity</td>
<td>36</td>
</tr>
<tr>
<td>1.3.5 Multivalent aptamers</td>
<td>38</td>
</tr>
<tr>
<td>1.3.6 Cell purification</td>
<td>40</td>
</tr>
<tr>
<td>1.3.6.1 Affinity-based Purification techniques</td>
<td>40</td>
</tr>
<tr>
<td>1.3.6.2 Clinical applications</td>
<td>41</td>
</tr>
<tr>
<td>1.4 Significance of this work</td>
<td>42</td>
</tr>
<tr>
<td>1.4.1 Novel metric for evaluating vascular graft performance</td>
<td>42</td>
</tr>
<tr>
<td>1.4.2 Aptamers selective toward LO-EPCs</td>
<td>43</td>
</tr>
<tr>
<td>1.4.2.1 Therapeutic utility</td>
<td>43</td>
</tr>
<tr>
<td>1.4.3.1 Diagnostic utility</td>
<td>45</td>
</tr>
</tbody>
</table>
1.4.3 Antidote-reversible aptamer stains for purification of natively functional cells .................................................................45

2. Coagulation-induced resistance to fluid flow in small-diameter vascular grafts and graft mimics measured by purging pressure ........................................................................................................47

2.1 Introduction ........................................................................................................................................................................47

2.2 Materials and methods .........................................................................................................................................................51

2.2.1 Materials ........................................................................................................................................................................51

2.2.2 Fractional occlusion of Tygon tubes ...............................................................................................................................52

2.2.3 Blood collection and plasma isolation ............................................................................................................................53

2.2.4 Fluid loading into ePTFE vascular grafts .......................................................................................................................53

2.2.5 Fibrin CTs ......................................................................................................................................................................55

2.2.6 PRP and blood CTs .........................................................................................................................................................55

2.2.7 Rheology of glycerin solutions, fibrin glue and blood ....................................................................................................56

2.2.8 Spectroscopic characterization of fibrin glue clotting reaction .....................................................................................57

2.2.9 Pressure measurement system ..................................................................................................................................57

2.2.10 Purging pressure measurements .................................................................................................................................59

2.2.11 Modeling theoretical flow resistances ..........................................................................................................................60

2.2.12 Statistics ....................................................................................................................................................................61

2.3 Results ..................................................................................................................................................................................61

2.3.1 Solution viscosity and fractional occlusion pressure measurements ........................................................................61

2.3.2 Fibrin glue and whole blood pressure measurements ................................................................................................62

2.3.3 Rheology of fibrin glue ....................................................................................................................................................66
2.3.4 Comparison of experimental results to theory..............................................67
2.4 Discussion........................................................................................................69
2.5 Conclusions ........................................................................................................76

3. Aptamers for the selective capture of late-outgrowth endothelial progenitor cells.....78
   3.1 Introduction......................................................................................................78
   3.2 Materials and methods ..................................................................................82
      3.2.1 Overview of nuclease-resistant RNA (nrRNA) subtractive cell-SELEX........28
      3.2.2 Isolation of LO-EPCs and buffy coat cells..............................................84
      3.2.3 Double-mutant T7 RNA polymerase (YFHA) production and activity assessment..................................................85
      3.2.4 Preparation of initial random nrRNA library ..........................................86
      3.2.5 Performing rounds of positive selection..............................................87
      3.2.6 Performing rounds of positive selection followed by negative selection.....90
      3.2.7 Increasing selection pressure to retain strongest binders.....................91
      3.2.8 Biotinylation and fluorescent labeling of aptamers...............................92
      3.2.9 Assessing enrichment via flow cytometry...........................................93
   3.3 Results ...........................................................................................................94
      3.3.1 Double mutant T7 RNAP (YFHA) production and activity assessment.....94
      3.3.2 Biotinylation assessment via EMSA ......................................................95
      3.3.3 Enrichment after five selection rounds ...............................................96
   3.4 Discussion......................................................................................................97
   3.5 Conclusions ....................................................................................................105
4. Antidote-mediated reversal of extracellular aptamer staining ........................................ 107

4.1 Introduction .................................................................................................................. 107

4.2 Materials and methods ............................................................................................... 113

4.2.1 Cell culture ............................................................................................................. 113

4.2.2 Chemical synthesis of aptamers ........................................................................... 113

4.2.3 Biotinylated aptamer transcription and aptamer-streptavidin conjugate preparation ......................................................................................................... 114

4.2.4 Electrophoretic-mobility shift assay (EMSA) .......................................................... 117

4.2.5 Cell staining and flow cytometry ........................................................................... 117

4.2.6 Apparent binding affinity determination .................................................................. 119

4.2.7 Fluorescence activated cell sorting ........................................................................ 119

4.2.8 Antidote synthesis and screening experiments ...................................................... 120

4.2.9 Destaining cells ...................................................................................................... 120

4.2.10 Cell viability assays .............................................................................................. 121

4.2.11 EGF stimulation assays and Western blotting ....................................................... 122

4.2.11 Statistics ................................................................................................................ 124

4.3 Results ............................................................................................................................ 124

4.3.1 Best performing antidotes targeted predicted loops of E07 .................................. 124

4.3.2 Antidote enhanced removal of bound E07 .............................................................. 126

4.3.3 2′OMe RNA antidote was more potent than DNA ............................................... 126

4.3.4 Antidote-enhanced destaining was specific ............................................................ 127
4.3.5 Formation of higher valency conjugates was favored by increased aptamer to SA ratios.......................................................................................................................... 129

4.3.6 Higher ratio E07:SA conjugate mixtures exhibited higher apparent affinities ........................................................................................................................................... 131

4.3.7 E07-SA conjugate mixtures were more stable than E07 during sorting and destaining .............................................................................................................................................. 132

4.3.8 High cell viability was preserved during sorting and destaining.................. 133

4.3.9 Destaining rescued native stimulability of aptamer-stained cells............... 134

4.4 Discussion...................................................................................................................... 139

4.5 Conclusions ................................................................................................................... 147

5. Conclusions and perspectives ............................................................................................ 148

5.1 Dissertation summary.................................................................................................. 148

5.2 Implications of research............................................................................................. 151

5.3 Future work.................................................................................................................. 155

Appendix A: License for previously published work ....................................................... 158

Appendix B: Proposed methods for evaluating LO-EPC aptamers ............................. 168

Characterization of LO-EPC aptamers ........................................................................ 168

Overall goal and approach............................................................................................. 168

Proposed methods........................................................................................................... 168

High-throughput sequencing (HTS) ............................................................................ 168

Identification of aptamer candidates using bioinformatics ...................................... 169

Assessment of aptamer binding affinities .................................................................... 169

Elucidation of aptamer targets (biomarker identification) ........................................... 171
Assessment of the ability of the aptamers to capture cultured and uncultured (circulating) LO-EPCs ...........................................................................................................172

Overall goal and approach ............................................................................................172

Proposed methods ..........................................................................................................172

Evaluating aptamer capture efficiency of cultured LO-EPCs ......................................172

Assessing utility of aptamers to capture uncultured LO-EPCs.................................174

References ......................................................................................................................176

Biography .......................................................................................................................203
List of Tables

Table 1: Sequences of aptamers and primers. ................................................................. 115

Table 2: Sequences of screened antidotes. ................................................................. 122
List of Figures

Figure 1: A graphical representation of circulating EPC levels as a function of vascular health as conceived by Fadini et al. EPC levels gradually decline during atherosclerotic progression, with levels declining faster and to a greater degree in individuals at higher risk for adverse cardiac events. Adverse events trigger mobilization of cells to repair vascular damage, but this repair mechanism is retarded in higher risk individuals. ............................................................................................................................... 23

Figure 2: An overview of subtractive whole-cell SELEX................................................................. 31

Figure 3: Influence of initial number of isolated late-EPCs on (1) duration to achieve full graft coverage and (2) risk of an interim cardiac event given this duration. A higher amount of starting cells greatly reduces the risk to waiting CABG patients. This work may significantly reduce this risk by reducing the lead time for a suitable coronary bypass graft................................................................................................................................. 44

Figure 4: Method used to make measurements with the devised system. (A) Air pressure was monitored proximal to tested vascular grafts via two types of pressure sensors to provide sensitive measurements over a larger dynamic range. Tygon grafts were attached to the vertically oriented system and solutions were drawn into mock grafts using a syringe pump. Alternatively, ePTFE grafts were manually loaded with solution and attached to the system (Figure 5). After allowing the solution to cure for an appropriate duration, graft contents were purged at a constant flow rate. (B) Resistance to flow while purging results in compression of air proximal to the graft until a maximum pressure is reached. This maximum pressure served as the primary end point for pressure measurements. ................................................................................................................................. 50

Figure 5: Method for loading clotting solutions into ePTFE vascular grafts. Grafts connected to male luer-lock tubing adaptors were oriented horizontally and then loaded with clotting solutions via a needled syringe. The distal end of the graft then was crimped with surgical ligating clips to prevent leakage upon orienting the graft vertically. After burping trapped air bubbles, clotting solutions were allowed to cure in a vertical orientation for desired durations................................................................................................................................. 54

Figure 6: Experimentally observed maximum purging pressures for a range of fluid viscosities and cross-sectional areas available for flow exhibited similar trends to theoretical flow resistances predicted by the electrical circuit analogy for Poiseuille’s law. (A) Greater values of solution viscosity and fractional occlusion resulted in greater
maximum pressures while purging Tygon grafts. Glycerin solutions ranging in viscosity from 1 to 1410 cP were purged from modified Tygon grafts with a range of percent occlusions, with each occlusion level significantly different from all others (p < 0.0001 except 40 vs. 60% at p < 0.05). More viscous solutions also generated significantly maximum higher pressures, with each tested viscosity significantly different from all others (p < 0.0001) except for 1 vs. 8.3 cP, which were statistically similar (p = 0.96). The inset provides a clearer view of the same data for fractional occlusions ≤60%. Data are mean ± standard error of the mean (SEM); n = 3 and small error bars are obscured by data points. (B) Theoretical flow resistances computed for corresponding experimental conditions possessed similar trends to measured data. .......................................................62

Figure 7: Maximum pressures were (1) maintained for longer upon purging nonporous Tygon grafts of fibrin glue or whole blood cured for longer durations (fractions of clotting time (CT) as noted) and (2) highly variable at the same curing duration upon purging blood and PRP from porous ePTFE grafts. Individual pressure traces (representative of multiple trials) generated while purging fibrin glue (A) and whole blood (B) from Tygon reveal that both the maximum pressure and the period for which that pressure persisted increased with greater curing times. While fibrin glue pressure traces were highly reproducible, blood traces were highly variable in Tygon at and just after the clotting time (two traces of 1.0 and 1.25 CT shown to illustrate variability). (C) Purging whole blood and PRP cured for their clotting times (1.0 CT) from ePTFE was even more variable, with purging pressures ranging from near baseline to ~140 mmHg (three traces of each clot type shown to illustrate variability). Purging blood cured in Tygon for 150% of the clotting time (1.5 CT; ~45 min) (B) and in one case PRP cured in ePTFE for 100% of the clotting time (run 3 of 1.0 CT; ~120 min) (C) resulted in maintenance of maximum pressure until data collection stopped (data not shown).......63

Figure 8: Higher maximum pressures resulted from purging fibrin glue and reactivated whole blood cured for longer durations from both Tygon and ePTFE grafts. Fibrin glue was allowed to cure for various durations normalized to its clotting time and then purged from both graft types, and same was done with blood in Tygon tubes. Curing of fibrin glue was also monitored spectroscopically (inset). Maximum pressures and solution turbidity (absorbance at 405 nm) both increased with increasing curing durations, with each curing duration significantly different from all others (p < 0.05 for *, **, *** and ****). However, no significant differences between the type of graft or clotting solution were observed for a given curing duration. Data are mean ± SEM; n = 4. ...........65

Figure 9: Highly variable maximum pressures were generated upon purging blood or PRP from ePTFE grafts at the clotting time. Reactivated blood and PRP were allowed to
Cure until the clotting time and then purged from ePTFE grafts. The clotting solutions were also purged just after reactivation for comparison. Maximum pressures generated from purging both whole blood and PRP were extremely variable, ranging from baseline pressure levels to nearly 140 mmHg (also shown in Figure 7(C)). Consequently, maximum pressures generated by purging blood and PRP at each duration were statistically similar (p = 0.71). Despite this variability, maximum pressures generated at the clotting time were significantly higher (p < 0.0001 for *) than those produced just after reactivation of blood or PRP. Data are mean ± SD; n = 10.67

Figure 10: The apparent viscosity of fibrin glue increased and became more variable as it cured up to and beyond its clotting time. (A) Beyond the clotting time, apparent viscosity increased more slowly and seemed to approach a plateau. Due to the high variability in measurements beyond the clotting time (>1.0), all apparent viscosities are statistically similar except for a significantly lower value at a curing time of 0.9 (p < 0.05 for *). Data are mean ± SEM; n = 3. (B) Maximum purging pressures increased faster and were less variable than apparent viscosity for corresponding curing times of fibrin glue (noted for each data point as fractions of clotting time). Statistics of pressure and apparent viscosity data are identical to those presented in Figures 8 and 10(A), respectively, and are omitted here for clarity. Viscosity data are mean ± SEM, n = 3, and pressure data are mean ± SEM, n = 4.68

Figure 11: Comparison of experimental and theoretical resistances generated by purging pure glycerin (gray line) and fibrin glue (black line) from Tygon tubes. The diagonal dashed line indicates perfect correlation of tube pressure described by Eq. (1) and the experimentally determined maximum purging pressures. Maximum purging pressure increased with percent tube occlusion and fibrin glue viscosity as theoretically predicted; however, maximum pressures generated when purging pure glycerin from the range of percent occluded tubes (Figure 6) were higher than predicted by theory, whereas maximum pressures generated when purging fibrin with a range of experimentally measured apparent viscosities from tubes (Figure 10(B)) were lower than predicted by theory.69

Figure 12: An overview of the proposed work. An iterative in vitro selection method called subtractive whole-cell Systematic Evolution of Ligands by Exponential Enrichment (SELEX) was used to evolve nuclease-resistant RNA (nrRNA)-based aptamers that bind LO-EPCs with high specificity from an initially random nucleic acid library containing ~10^15 unique oligomers without a priori knowledge of the binding targets. nrRNA pools from later selection rounds was then to undergo high-throughput sequencing to identify aptamer candidates with unique sequences. Aptamer candidates
were then to be individually tested for their apparent binding strengths to LO-EPCs from various donors and buffy coat cells. The binding targets on LO-EPC surfaces of the most selective aptamers were then to be elucidated by mass spectrometry to potentially reveal novel and uniquely characteristic LO-EPC surface markers. Finally, the utility of the generated aptamers to selectively capture both cultured and circulating (non-cultured) LO-EPCs using aptamer-conjugated magnetic beads was planned to be evaluated. ................................................................. 81

Figure 13: The particular subtractive whole-cell SELEX procedure used to generate nuclease-resistant RNA (nrRNA) aptamers selective for LO-EPCs amongst all other buffy coat cells. Details of this protocol are provided below. ................................................................. 83

Figure 14: Generation of the starting nrRNA pool containing ~10^{15} unique sequences. (A) ~10^{15} unique ssDNA oligomers (dark green) were annealed to primers (purple) containing a T7 RNA polymerase (RNAP) promoter (orange) and resultant 5’ overhangs were “filled in” by Klenow fragment to generate a random dsDNA pool. (B) The dsDNA pool was transcribed to a random nrRNA pool (red) using the double mutant T7 RNAP (YFHA) and a dNTP mix containing 2’OMe-substituted pyrimidines. After the transcription reaction, free DNA and residual heteroduplexes were digested using DNase I to yield only nrRNA. .................................................................................................................. 87

Figure 15: PAGE purification of transcribed nrRNA pools. Generated nrRNA pools were first cleaned by organic extraction with 1:1 phenol/chloroform, concentrated and then ethanol precipitated. The pool was then purified on a large-format 15% denaturing polyacrylamide gel and the band of interest (71 bp) were visualized by UV shadowing, excised, crushed and extracted into TE buffer. Extracted nrRNA was separated from gel fragments and again concentrated to generate the pure nrRNA pool for the next selection round. .................................................................................................................. 88

Figure 16: Positive selection against cultured LO-EPCs (all rounds). LO-EPCs previously isolated from CAD patients were grown to ~85% confluence in TCPS petri dishes, washed 3x with wash buffer, and a solution containing a pool of nrRNA (~10^{15} in round one, ~10^{14} in subsequent rounds) in binding buffer was gently rocked over the cells. After this binding reaction, unbound and weakly bound oligos were removed via washing several times. The conditions of the binding reaction and following washes were manipulated to retain only the strongest binders as the selection progressed. Cells were then detached by scraping and organically extracted in phenol/chloroform to elute binders (and endogenous nucleic acid). Isolated nucleic acid was then cleaned up and
prepared for reverse transcription. This was performed in all rounds and directly followed negative selection in even-numbered rounds. ...............................................................89

Figure 17: Negative selection against freshly isolated buffy coat cells (even rounds). Freshly collected whole blood is fractionated by density gradient centrifugation and buffy coat cells are isolated by pipetting. This population was washed into binding buffer and 10 M cells were combined with 200 pmol nrRNA pool from the last selection round. This binding reaction was carried out with agitation to maintain the cell suspension at 4°C for an hour. Cells were then pelleted by centrifugation to partition and isolate non-binders of interest in the supernatant, which are then combined with an appropriate volume of binding buffer and directly transferred onto LO-EPCs for positive selection. In later rounds (6-8) of selection, buffy coat binders were eluted and converted to dsDNA as the round-specific “negatively selected pool” for later use in HTS and bioinformatics. ............................................................................................................91

Figure 18: Selection conditions chosen to increase selection pressure through round 8. Throughout the selection (1) binding reaction and wash conditions became more stringent to retain only the strongest binders and (2) various LO-EPC lines at a range of “ages” and different buffy coat cell sources were used to subtract out binders to any donor-specific markers. Only LO-EPCs isolated from CAD patients were be used for the selection........................................................................................................................................92

Figure 19: Procedure used to stain cells with fluorescently labeled aptamers for enrichment monitoring via flow cytometry. 5'-biotinylated nrRNA pools from select rounds were labeled with a streptavidin-PE conjugate and then incubated with LO-EPCs (shown) or buffy coat cells under the binding reaction conditions of the last performed round. Cells were then washed and stored on ice until flow cytometry. .......................94

Figure 20: Recombinantly produced YFHA enzyme was pure exhibited good activity. (A) SDS-PAGE showed that several dilutions (lanes 2-6) of YFHA stock were exceptionally pure, with only a single band of expected size (100 kDa) even when loading the greatest mass of product (lane 2). (B) Activity was assessed by performing transcriptions with commercially available non-mutant T7 RNAP and in-house produced YFHA. Both enzymes had similar trends, with activity dropping off after a couple of hours and overall lower apparent activity for the native enzyme at the added concentration of 20 U / 100 μL. YFHA activity was essentially the same transcribing both native and nrRNA............................................................................................................95
Figure 21: Biotinylation of nrRNA was successful. A gel-shift assay using denaturing PAGE revealed that migration of biotinylated but not regular nrRNA was retarded when pre-reacted with streptavidin (SA), indicating that biotinylated nrRNA was complexed with SA and consequently migrated based on the mass of the complex rather than the nrRNA alone. .................................................................96

Figure 22: Flow cytometric analysis indicated loss of initially observed LO-EPC-specific enrichment by round 5. Binding capacity of nrRNA pools toward LO-EPCs increased through round 3 (R3; light green trace) but decreased to that of the random starting pool (R0, light blue trace) by R5 (pink trace), indicating a drop in the apparent mean affinity of the pool beginning at R4 (dark green trace). These differences are better observed and compared touffy coat cells in Figure 24.................................................................97

Figure 23: Flow cytometric analysis of multipleuffy coat revealed undesirable enrichment. The entireuffy coat population was gated into three subpopulations as indicated and round-specific per-cell fluorescence was plotted. Binding capacity of nrRNA pools towarduffy coat cells was not substantially changed through R3, but significantly increased in R4 (“main” and “minor” subpopulations) and R5 (all subpopulations). These differences are better observed and compared to LO-EPCs in Figure 24.................................................................98

Figure 24: Binding capacity toward LO-EPCs increased through R3, then decreased concomitantly with increased binding capacity towarduffy coat cells in R4 and R5. (A) Raw mean fluorescent intensities (MFI) of each cell population across all rounds (R0-R5) including unstained cells (“none” – no nrRNA added). Per-cell fluorescence increased for LO-EPCs through R3 and then decreased in subsequent rounds. Substantial absolute fluorescence of cells in the “major”uffy coat population was seen, indicating a high initial binding capacity for this subpopulation that was preserved through R4 and significantly increased R5 to greater than that for LO-EPCs. (B) Enrichment relative to the unselected starting pool over five rounds. Moderate enrichment for LO-EPCs was observed by R3, but decreased in later rounds as enrichment for alluffy coat cells was observed. (C and D) Relative per-cell fluorescence (C and D are inverses; both shown for clarity) indicate the relative number of binders per cell and provide a sense of specificity of the overall pools. Greater LO-EPC specificity achieved by R3 decreased in later rounds.................................................................99

Figure 25: Overview of using an aptamer to selectively stain and sort target cells followed by antidote treatment to remove the aptamer and restore native cell function. Target cells of an initially heterogeneous population are selectively labeled with a
surface receptor-specific aptamer (A) and then isolated via fluorescence-activated cell sorting (FACS) (B). In the case of neutralizing aptamers and antibodies, the lingering stain on purified target cells can inhibit native receptor function (C) and confound downstream research and clinical applications. However, subsequent treatment of aptamer- (but not antibody-) stained cells with matched antidotes (D) allows gentle stain removal, restoring native receptor function (E) and enabling intended use of the target cells. The presented work demonstrates proof-of-principle of this technology using the AF488-labeled neutralizing EGFR-binding aptamer E07 and paired antidotes to stain, sort and “destain” epidermoid carcinoma (A431) cells ahead of probing targeted receptor function. 

Figure 26: Predicted secondary structure of E07. One of two secondary structures predicted to be highly favorable and dominate when folding at 4°C, temperature at which this work is carried out except for the 37°C treatment to destain cells. This structure is also predominantly predicted by the software at 37°C.

Figure 27: The designed 5’ 8 base-long tail of E07 (TbE07) does not impact predicted secondary structure. The structure of E07 predicted at both 4°C and 37°C was maintained after addition of a tail to its 5’ end. This extension was added to facilitate SA conjugation.

Figure 28: Antidote screening targeted entire sequence of E07. Fifteen base-long DNA antidotes (A1 – A17) were used to probe nearly the entire length of the E07 sequence in two-based increments. Antidote target regions are highlighted in green, and the predicted loop-forming regions often critical for aptamer binding are bolded. The 3’ “T” denotes an inverted thymidine added for aptamer stability that is present only on chemically-synthesized E07.

Figure 29: Antidote screening revealed loop-targeting antidote A9 as most promising candidate. A431 cells were stained for 30 min with C36 (random control RNA) or E07 in either the absence or presence of 1000-fold molar excess (100 μM) of antidote at either 4°C or 37°C. Samples stained in the absence of antidote were subsequently washed and resuspended in antidote under the same conditions as for staining. Unstained, C36-stained and non-antidote treated E07-stained cells served as controls. Washed samples were then analyzed by flow cytometry and the fraction of the meant fluorescence intensity (MFI) relative to the non-antidote treated E07 samples were plotted. A reduction in MFI indicated antidote-mediated blocking of aptamer binding or removal of bound aptamer. Staining both in the (A) presence and (B) absence of antidote at 4°C only modestly reduced the amount of bound E07, with antidotes targeting predicted
loop region being most effective. However, staining in the presence of antidote at 37°C (C) dramatically reduced the amount of bound E07 using antidotes that targeted predicted loop regions. (D) Antidote A9 emerged as the best candidate from these screening assays. Its targeted and was used for subsequent experiments. .......................... 125

Figure 30: Antidote A9 enhances removal of E07 from cells over media only at 37°C. E07-stained A431 cells resuspended in either media or antidote (100 μM A9) incubated at 37°C removed bound E07. Greater destaining was achieved with longer durations at 37°C both cases, but antidote greatly increased the rate and amount of E07 removed compared to media only. .......................................................................................................................................................................................................................... 127

Figure 31: Antidote mA9 (2′OMe RNA) is more effective than A9 (DNA) at lower concentrations. The potency of DNA-based antidote A9 and its 2′OMe-based counterpart mA9 were compared in a titration experiment. E07-stained A431 cells were resuspended in media containing A9 or mA9 in concentrations ranging from 50 nM to 25 μM and incubated at 37°C for 10 min. Destained cells were washed, analyzed by flow cytometry and the relative reduction in fluorescence corresponding to fold greater E07 removal was plotted for each antidote concentration. Flow curves for (A) A9-treated and (B) mA9-treated cells show that while both antidote types offered similar performance at higher concentrations (≥ 5 μM), mA9 removed substantially more bound E07 at lower concentrations (< 5 μM). The higher potency of mA9 is well visualized by examining the fold greater E07 removal as a function of antidote concentration (C)..... 128

Figure 32: Antidotes A9 and mA9 specifically enhance E07 removal. Non-specific destaining was assessed by comparing the effectiveness of A9 and mA9 to a random-sequence control antidote (sA9). E07-stained cells were incubated with media only or each antidote type at 37°C for various durations up to 10 minutes, washed and analyzed by flow cytometry. The reduction in fluorescence corresponding to removal of bound E07 over time is shown in (A), and the enhancement of E07 removal over media only is highlighted in (B). Even at the high antidote concentration of 25 μM, the control antidote sA9 did not improve removal of bound E07 over media only. ................................. 129

Figure 33: Higher valency aptamer-streptavidin conjugates were generated using higher molar ratios of aptamer to streptavidin. The valency of aptamer-streptavidin (SA) conjugates prepared with different molar ratios of biotinylated aptamer to SA was characterized by electrophoretic mobility shift assays (EMSAs). C36 or E07 with 5’ biotinylated tails (TbC36 and TbE07) was reacted with tetrameric SA with four biotin binding sites in 1:1, 2:1, 3:1 or 4:1 molar ratios of aptamer to tetramer. Free RNA (1:0) and conjugate samples containing equal amounts of SA were then separated by
Figure 34: Higher ratio E07:SA conjugate mixtures possessed higher apparent affinities but lower apparent saturation. A431 cells were stained with various concentrations of E07 only or E07- SA conjugate mixtures prepared by reacting 1:1, 2:1 or 3:1 biotinylated E07 with tetrameric streptavidin. Stained cells were washed, analyzed by flow cytometry and their fluorescence corresponding to the amount of bound E07 or E07-SA was plotted to determine apparent binding affinities (dissociation constants; KD) and cell surface saturation (total number of receptors; Bmax). Conjugate mixtures prepared from higher E07:SA ratios had higher apparent affinities, but lower apparent saturation. Unconjugated E07 had a higher affinity than the 1:1 conjugate mixture but lower affinity than the 2:1 and 3:1 mixtures........................................................................................................ 130

Figure 35: Significantly more monomeric E07 than E07-SA conjugate mixture was lost during sorting. Stained A431 cells were analyzed by flow cytometry immediately before and after FACS to examine losses that occurred while sorting. E07 was the least stable with losses of 46%, followed by E07-SA (2:1 E07:SA conjugate mix) with 25% losses and EGFR antibodies with about 11% losses each. Each group is significantly different from all others (p < 0.0001 for *, ** and ***)......................................................................................................... 132

Figure 36: Destaining sorted cells highlighted additional stability of E07-SA conjugate over E07 monomer. A431 cells stained with E07, E07-SA (2:1 E07:SA ratio conjugate mixture) or one of two different monoclonal EGFR antibodies were sorted by FACS and then destained in media, 500 nM mA9 or 5 μM mA9 at 37°C for up to 30 min to remove bound E07 or E07-SA. Cells were then analyzed by flow cytometry and reduction in fluorescence of each stain type corresponding to its removal was plotted. EGFR antibodies ICR10 and D1D4J were the most stable stains, experiencing only slight losses

xxiv
over 30 min at 37°C in media (A). E07-SA was substantially more stable than E07 both in media only and in antidote, but higher concentration of antidote (5 μM vs. 500 nM) effectively removed the bound conjugate mixture over 30 min (B and C).

Figure 37: High cell viability was maintained after sorting and destaining for all treatments. Live/dead assays indicated that high A431 cell viability was maintained regardless of staining and destaining treatment. Prior to sorting cells were either left unstained or stained with EGFR antibody (D1D4J), C36, E07, SA-C36 (2:1 C36:SA conjugate mixture) or E07-SA (2:1 E07:SA conjugate mixture). Sorted cells were then destained for 0 or 30 min in media only or 5 μM mA9. Unstained, sorted cells that were untreated or heat-treated (65°C for 15 min) respectively served as (A) live and (B) dead controls. No treatment significantly impacted viability relative to the live control, with all conditions sustaining viabilities >95% (C and D).

Figure 38: Only destaining with antidote restored ability of unsorted aptamer-stained cells to be stimulated with EGF. Quantitative Western blotting enabled quantification of EGF-induced stimulation of A431 cells via probing for phosphorylated (pEGFR; red) and total EGFR (green). * denotes stimulation with EGF; media DS = destained with media only; mA9 DS = destained with 5 μM mA9. Antibody and unstained cells were destained with media only. The degree of stimulation was taken as the ratio of the band intensities of pEGFR and total EGFR, as EGF binding triggers autophosphorylation of EGFR to generate pEGFR. Stained or unstained cells were destained for 5 min at 37°C in media or 5 μM mA9, stimulated with 5 nM EGF for 15 min on ice and then lysed in the presence of phosphatase and protease inhibitors. Crude lysate samples containing equal amounts of protein were prepared, separated by denaturing polyacrylamide gel electrophoresis and blotted onto a membrane. The membrane was blocked with albumin and incubated overnight at 4°C with primary antibodies for total EGFR and pEGFR. Secondary antibodies were used for detection and the blots were imaged. Band intensities corresponding to total EGFR pEGFR were quantified by software and the pEGFR/total EGFR ratio for each sample was normalized to the average ratio for stimulated unstained cells, which served as the positive control for native, uninhibited stimulation. Destaining (A) E07 and (B) E07-SA (2:1 E07:SA conjugate mix) with media yielded significantly reduced stimulation compared with unstained cells. However, destaining with mA9 removed enough (A) E07 and (B) E07-SA to enable native stimulation levels in line with those of unstained cells. The neutralizing EGFR antibody D1D4J irreversibly suppressed stimulation to a greater degree than any other treatment (B). Each group is significantly different from all others (p < 0.0001 for *, **, *** and ****).
Figure 39: Antidote treatment was necessary after sorting for restored EGF stimulability of E07-SA-stained but not E07-stained cells. Quantitative Western blots were also performed on A431 cells that had been sorted via FACS. Destaining, stimulation, lysing and Western blotting procedures to probe for phosphorylated EGFR (pEGFR; red) and total EGFR (green) were identical to those used for unsorted samples. * denotes stimulation with EGF; media DS = destained with media only; mA9 DS = destained with 5 μM mA9. Antibody and unstained cells were destained with media only. Destained C36 and SA-C36 (2:1 C36:SA conjugate mix) samples (A) did not inhibit stimulation of cells relative to the unstained positive control, but C36 and SA-C36 samples did stimulate cells to slightly different degrees (p = 0.012 for ** vs. ***). Unlike unsorted samples, destaining sorted E07 samples (B) with media was sufficient to restore stimulation to native levels. In contrast, sorted E07-SA samples (2:1 E07:SA conjugate mix) (C) still required antidote to rescue cell stimulability.

Figure 40: Algorithm to filter potentially important aptamer sequences from HTS data. Erroneously sequenced oligos from selection pools should be removed from data to leave correct sequences of full-length reads. This filtered pool should then be processed to enumerate and rank the frequency of unique sequences in pools from positive selection relative to pools from negative selection (subtracted sequences) for a given round. Unique sequences most enriched in the positive pool relative to the negative should be classified as potentially important aptamer candidates.

Figure 41: The magnetic bead-based method to assess capture efficiency of LO-EPCs aptamers. SA-coated magnetic beads should be pre-reacted with 5'-biotinylated aptamers, washed to remove residual nrRNA and combined with mixtures of LO-EPCs doped into well-defined buffy coat solutions (depicted) or whole blood. Captured cells should be isolated from non-bound cells by application of a magnet, liberated via trypsinization and cultured prior to characterization.
Acknowledgements

This chapter of my life has been rife with twists, turns and several cliffhangers for those watching as well as myself. I have so much gratitude for the generous professional and personal support offered by many kind souls along this journey. To Monty – my advisor and the humble man who took a chance on this kid – thank you for being in my corner and being so deeply understanding through the many tough times. You’ve done right by me when you didn’t have to, and you’ve always given it to me straight. I will always appreciate your having kept the picture big – like, really big. To the rest of my original committee – Charlie, Bruce K., Becke and Tom Povsic – thank you for patiently having stuck with me and providing honest guidance (to which I should have better adhered) and wise perspective on both research and life. To Tom and Becke in particular who told me what I needed to hear and helped me find a new home when I was a stray in purgatory - thank you so much for your guidance and reaching out on my behalf. Last but definitely not least, a huge thank you goes out to Bruce S. who was willing to take this old dog and teach him new tricks. You even provided me with a doggie bed and a place to do my work digging in the yard. Thank you so very much for helping me see this through and making the ending a happy one. Namaste.

I’ve made many close friends along the way in the BME department that have warmed my heart and helped keep my head high and right – too many to name all here.

xxvii
To Suzana, my hilarious lab buddy who was there for the ups, downs and all-rounds – thank you for being you. Your company made the lab a special place. To Vrad – you showed me what true strength is, and not just pertaining to Krav Maga or tree fighting. Thanks for being a model of perseverance and your ever-sage council. And to Dave, the gene genie, thanks for being a great friend and telling me what I needed to hear when I needed to hear it – including when massive TVs were on sale at Amazon. I also want to give special shout outs to Mike Desoto, Marcus Henderson, Matt Brown, Mike Cook and Bin Li who lent their ears to my whining and slapped me back to reality when needed. Your jokes, kindness and willingness to help with whatever whenever gave me some hope for humanity after all, even though the robots will surely still win in the end.

Why does the whole Sullenger lab have to be so awesome? You’re going to bust my keyboard. Julie – your witty, raw, no BS-osity is inspiring and keeps the gears of the lab turning. Kady-Ann – thanks for the goodies (beer, rum cake) and making me feel a little less OCD by comparison. Ruwan – thanks for your bloody stories, encouragement, support and perspective, especially on Marvel movies. Janoo – thanks helping me embrace my inner child that lurks just beneath the surface – you are hilarious. Korie – are you done yet? To George – thank you so very much for taking care of Olive and being such a caring person. Our geek-out times are only topped by our nerd-out ones, and I’ve never met anyone who can find deals and sniff out scams quite like you.
Linsley – thanks for being so kind and helpful when it comes to both data analysis and wine suggestions. You see Humvees where no one else can. Angelo – thank you for being so real, genuine and compassionate. Your companionship, always-open door and music suggestions have really helped me steer the boat through the night. And to Bethany – what can I say? Thank you for everything. You’ve shared your stuff, space, time and literally an entire project with me. You’ve been so understanding and encouraging, and I couldn’t have done without you. To the whole lab – thank you for taking me in and making me one of your own. The sense of belonging and community you’ve shared helped me get back on track and over the finish line. You’re some of the finest people I’ve ever met, figuratively and figuratively. Thank you for your collective mentorship and friendship.

Mom and Dad – thanks first and foremost for having me; being alive is pretty great. No matter how things might turn out along this winding adventure, you’ve been supportive and reassuring. Turns out you were right – I may actually get a real job one day. To Dad in particular – your insane work ethic has always been a wonder to behold, but these past few years throughout your recovery it’s been truly inspiring. You don’t know how to give up, and I am fortunate to have had some of that rub off on me. It’s an honor to be your son. And to Mom – you’re one of the strongest people I know, and you always put others first. Thanks to you both for being there for me.

xxix
And to my family – Wendy, Olive and Louie – you are my rocks and make it all worthwhile. Your unwavering patience, love and support have made me both feel and actually (probably) be the luckiest guy ever.

xxx
1. Introduction

1.1 Synthetic vascular grafts

1.1.1 Clinical significance of high-performance synthetic vascular grafts

Coronary artery disease (CAD) is one of the leading causes of death worldwide. Domestically, the disease is responsible for one in five adult deaths and killed more than 600,000 people in 2006. CAD results from the progressive buildup of fatty deposits (plaques) that narrow and ultimately block the coronary arteries, increasing the likelihood of both (1) heart attack and (2) heart failure over the long-term. Coronary artery bypass grafting (CABG) surgery is necessary to circumvent blocked vessels and restore blood flow, and unfortunately CAD patients commonly require repeat operations over their lifetimes. Autografts utilizing saphenous veins or internal mammary arteries have historically been quite successful and are thus traditionally the first choice as vessel sources.

However, in many cases patients do not possess autologous vessels suitable for grafting, and the situation dramatically worsens with each repeat operation. Thus, there is an immense and urgent need for small-diameter autologous graft alternatives which match as closely as possible the native biochemical and mechanical properties of blood vessels. Tissue engineering may eventually deliver viable vessels custom-tailored to individuals and with near-native characteristics, but widespread clinical implementation...
is likely still decades away. In contrast, synthetic vascular grafts represent an immediately available solution as they have been extensively and successfully used for years. Regrettably, small diameter (<6 mm) synthetic grafts appropriate for coronary bypass suffer from poor patency rates resulting from thrombosis and neointimal hyperplasia.

A promising solution to these issues is forming a lining of endothelial cells (ECs), which possess intrinsic anti-thrombogenic and anti-hyperplasia characteristics, within these grafts. Endothelialization has improved synthetic graft performance and mitigated the severity of thrombosis and hyperplasia, but EC sourcing is non-trivial. Allogenic sourcing would facilitate development of off-the-shelf solutions, but this is a logistically difficult due to the requirement for HLA-matched donors in order to avoid transplant rejection. Autologous EC sourcing imposes substantial risk on waiting CABG patients due either (1) an invasive pre-CABG surgery to harvest ECs from fat or vessels or (2) a prohibitively long, morbidity-associated wait time to amass sufficient quantities of non-invasively acquired ECs resident in peripheral blood. Methodologies enabling rapid preparation of endothelialized synthetic vascular grafts would have tremendous clinical impact by addressing the pressing need of feasible substitutes for natural blood vessels.
1.1.2 Vascular grafts failure modes

Reduced patency and ultimately failure of both natural and prosthetic vascular grafts primarily results from thrombosis and/or neointimal hyperplasia. Occurrence in prosthetic grafts is mostly due to non-native surface chemistries and mechanical properties of these conduits.

Neointimal hyperplasia is a complicated cellular and molecular response characterized by SMC proliferation. It is thought to arise from poor hemodynamics caused by a compliance mismatch between the prosthetic graft and adjacent native vessels. These altered hemodynamics generate non-native shear stresses, and it is thought that hyperplasia occurs as part of a long-term feedback loop to restore the “optimum” shear stress on the vessel lumen. As this tissue growth proceeds, graft patency will be reduced – especially in small diameter conduits with much smaller cross-sectional areas.

Thrombosis occurs due to activated platelet aggregation that eventually forms a fibrin clot. Further, platelet aggregation occurs more readily under laminar flow such as that experienced in the coronary arteries, considerably increasing the likelihood of thrombus formation. In synthetic vessels, platelets are usually activated by interaction with the bare material surface or deposited collagen. Once formed, thrombi have two general fates: (1) retention on the vessel/graft lumen, reducing patency, or (2)
detachment and mobilization into the bloodstream to form an embolism. Either of these situations is obviously undesirable and, along with intimal hyperplasia, negatively impact synthetic graft performance as well as increase the risk of additional medical complications.

1.1.3 Graft material treatments and coatings

One approach to achieve long-term graft patency is by surface modification of synthetic grafts to reduce thrombogenicity and neointimal hyperplasia. The techniques applied broadly fall into chemical and biological coatings. Chemical coatings do not include biological molecules such as proteins, focusing rather on incorporation of synthetic molecules to alter surface properties. Impregnation of carbon into graft materials is a popular strategy to reduce the chemical reactivity of the material and has been associated with reduced thrombogenicity in a host of biomedical applications \(^{17, 18}\). This process may additionally increase the electronegativity of surface, thus repelling platelet adhesion \(^{19}\). Grafts of ePTFE impregnated with carbon have performed both the same as and better than untreated grafts in different clinical trials \(^{17, 19}\). Plasma treatment of materials using radio frequency glow discharge has also been attempted with some promising results. For example, platelet adhesion and spreading was significantly reduced on treated Dacron in an ex vivo baboon shut model, which could translate into reduced thrombogenicity in vivo \(^{20}\). Another interesting development was the use of 2-
methacryloyloxyethyl phosphorylcholine (MPC) phospholipids to create small diameter vascular prostheses that displayed excellent anti-thrombogenicity and no intimal hyperplasia during an 8-week in vivo study in rabbit carotid arteries 21. Applied as a lumenal coating, this technology may improve long-term patency of vascular grafts. To specifically inhibit neointima formation, drug-coated synthetic grafts have been developed and are quite successful. Paclitaxel and sirolimus coatings on ePTFE grafts significantly decreased intimal hyperplasia in pigs compared with controls 22, 23. Delivery of nitric oxide (NO) is also being actively pursued as it is a natural inhibitor of platelet aggregation and intimal hyperplasia in native vessels 24. Though promising advances in NO generating molecules and their attachment to synthetic graft materials have been made, in vivo studies have not yet been published in the literature. As a whole, these chemical coatings provide passive inhibition of thrombus formation and in some cases neointimal hyperplasia. Biological coatings, on the other hand, provide the possibility of active thrombosis inhibition that may prove more effective in maintaining graft patency.

One of the simplest and oldest uses for biological coatings on synthetic grafts was to “pre-clot” them with blood in attempt to prevent seepage from woven or knitted Dacron grafts 25. Regrettably, this did not decrease thrombogenicity and was even shown to increase thrombogenicity 25. A popular and effective anti-thrombogenic
biological coating is heparin – a well-known, clinically used and powerful anticoagulant that prevents both the formation and growth of thromboses. As a coating, it is generally covalently bound to the graft surface due to safety concerns arising from the possibility of release into the bloodstream. In one study, covalently secured heparin on ePTFE prosthetic grafts delivered good patency rates in clinical trials 26. Another strategy employs heparin along with thrombomodulin (TM), a membrane-bound protein possessing both anti-thrombotic and anti-inflammatory properties, to greatly reduce thrombus formation in a flow circuit model 27. By itself, TM also successfully inhibits the coagulation and complement cascades in vitro as well as intimal hyperplasia on ePTFE stent grafts in vivo 28,29. Tissue plasminogen activator (tPA) is another anticoagulant protein that has been investigated as a biological coating for grafts. The protein catalyzes formation of plasmin, which is the primary enzyme that degrades thrombi 30. Coating ePTFE grafts with tPA was demonstrated to increase patency in vivo relative to control groups in one study, but few other synthetic graft studies with the enzyme have been published 31.

Many of the above-mentioned chemical and biological coating strategies are promising and may eventually lead to improved patency rates and in turn better small-diameter prosthetic graft performance. However, few clinical trials have been performed successfully and in many cases the reduction in graft lumen thrombogenicity
is meager at best. Additionally, concern has been raised over the longevity of chemical and especially biological coatings, which can undergo degradation and may lose their effectiveness over time. Further, some of the most effective agents to reduce thrombogenicity and intimal hyperplasia only attenuate one of these problems, requiring the use of multiple factors to improve patency rates. Nature has already evolved an all-in-one, superior solution in native vessels to regulate thrombus and neointima formation – the endothelium. Thus, formation of a viable and healthy endothelium lining on the lumen side of synthetic vascular grafts is an extensively pursued objective that, if achieved, could radically improve the patency rates of small-diameter synthetic grafts.

1.1.4 Endothelialization

The endothelium is a critically important continuum of ECs that serve many important functions in native blood vessels. Roles most pertinent to the improving vascular graft performance include (1) maintaining a non-thrombogenic surface, (2) regulating SMC proliferation, and (3) controlling immune cell attachment to the vessel lumen. As discussed above, (1) and (2) have been attempted with acellular graft coatings that strive to mimic some functions of the endothelium using select drugs or proteins. In fact, many of the proteins used in biological coatings are anti-thrombogenic factors innately and constitutively expressed by ECs. A clearly preferable alternative to
coatings is to establish a fully functional and persistent lining of ECs on the lumen side of the graft, which has already been proven to improve patency rates with promising results.

Clinically, there are two classic strategies to endothelialize grafts: a one-stage method, in which the graft is seeded with cells during the bypass procedure, and a two-stage method, wherein the graft is seeded and then cultured in vitro prior to implantation. In both cases, autologous ECs must be used to avoid an immune response and rejection. Trials using the one-stage method have not been encouraging, with graft patency rates being similar to bare grafts. The one-stage method is results in a lower density of ECs on the graft, and without coatings that augment cell adhesion strength the bulk of adhered cells are quickly sheared off when exposed to pulsatile flow. Generally, the number of adhered ultimately levels cells levels off within 24 h. The severity of this issue has been documented by one group that noted only 4±3% EC retention after one day. As a result, the two-stage method, which has demonstrated improved prosthetic graft patency over bare grafts in clinical trials, is generally preferred. This method involves harvesting ECs from an appropriate source, culturing and expanding them on the graft in vitro, and then implantation into the patient. Culture of the ECs for a few weeks allows for occupation of the graft by a
relatively dense population of cells, and this high-density seeding is commonly known as sodding\textsuperscript{39}.

Though sodding grafts has improved small diameter graft patency in animals and humans, many cells still wash off under flow and thus EC adhesion is not optimal. Endothelialization to improve graft performance is most effective when a continuous monolayer of ECs lines the graft lumen. Loss of cells may introduce discontinuities in the EC monolayer, exposing the potentially pro-thrombotic graft surface. Cell adhesion is dependent on a multitude of factors, including but not limited to: (1) surface modification of the graft (e.g. with coatings), (2) the type/source of ECs, (3) cell harvesting/culturing technique, and (4) flow conditioning of ECs. Additionally, sodding sometimes requires prohibitively large quantities of cells such that EC sourcing can become an issue. Care must thus be taken in generating and following an endothelialization protocol in order to maximize EC adhesion and viability. Some strategies to accomplish these goals are discussed in the following sections.

1.1.4.1 Endothelial cell (EC) attachment to graft surfaces

A number of strategies to improve EC adhesion to graft materials have been tried with varying degrees of success. Most bioactive coatings consist of extracellular matrix (ECM) proteins that present integrin-binding motifs with which ECs normally make focal contacts. Unsurprisingly, such coatings performed quite well on eTFE, Dacron, and
PU. These coatings were comprised of species such as collagen, fibronectin (FN), laminin, gelatin, and combinations thereof with generally increased EC attachment and/or retention under flow 40. Grafts pre-clotted with blood or coated with plasma proteins were also compared and enhanced endothelialization relative to bare grafts and in some cases relative to ECM component-coated grafts 40. Though it’s unclear which coating is the best for long-term EC retention, one study showed that coatings of laminin alone or with type IV collagen were superior in decreasing platelet accumulation relative to those of whole blood or especially FN 41. Clearly, some type of bioactive coating should be utilized to improve attachment of cells seeded onto prosthetic grafts.

More sophisticated systems to improve EC adhesion and retention have also been developed. One group has rigorously investigated and refined dual-ligand systems to improve EC attachment to surfaces. The rationale behind this method was to stabilize initial cell-surface attachment using a very high affinity interaction, such that cells could more easily establish native integrin-mediated focal adhesions with a substrate 42. This was approached in vitro by decorating ECs with streptavidin prior to flowing them over surfaces containing either biotinylated albumin and FN or biotinylated FN 42,43. This promising system improved cell adhesion and increased the density of focal adhesions formed relative to FN-coated surfaces alone, but unfortunately its effectiveness in vivo has not yet been probed.
Conditions under which ECs are harvested and cultured can also influence their attachment to surfaces. Harvesting veins for use as EC sources under so-called “gentle” conditions – in which the vein is excised and handled with care beyond that conventionally exercised – has been demonstrated to improve the rate and confluence of endothelialization of synthetic grafts 44. Simply culturing cells prior to use rather than using fresh cells improves their adhesion and the efficiency of graft seeding, providing another incentive to use a two-stage endothelialization method 45. Additionally, an incredibly simple yet very effective technique discovered to improve EC adhesion is the use of relatively mild trypsinization conditions during culture 46. Trypsin degrades membrane-bound integrins that bind motifs on surface-bound proteins, permitting detachment for passaging. Reducing the severity of the trypsinization conditions preserves more integrins, which allowed cells to rapidly and firmly adhere to FN-coated glass and FN-coated Teflon under flow inducing greater shear stresses than experienced in vivo.

Exposure to shear stresses generated by laminar flow is critical for maintenance of a healthy EC phenotype in vivo, and this influence has also been observed with ECs cultured in vitro. In native arteries, laminar flow generating a shear stress of 10-20 dyne/cm² usually results in quiescence and expression of anti-thrombotic factors by ECs, whereas deviation from these hemodynamics can result in phenotypic drift toward a pro-thrombogenic state 47. It is also important for ECs populating a prosthetic vascular
graft to maintain an anti-thrombotic phenotype. To achieve this, cells are often “flow conditioned” by being subjected to laminar flow over a range of shear stresses prior to or after seeding into grafts [47, 48]. Despite dissention over the optimal duration of flow exposure, it is widely accepted that flow conditioning is beneficial for EC retention and leads to a more “healthy” phenotype [35, 48, 49].

1.1.4.2 EC sources and types

Humans have a variety of different sources from which autologous ECs can be isolated for subsequent use in graft seeding. The most common EC sources used for endothelialization are native blood vessels (veins and arteries) and fat (omentum and subcutaneous) [50]. Regardless of the source, ECs need to be isolated by enzymatic breakdown of surrounding tissue followed by isolation of ECs. Clinical trials of grafts seeded with cells from these sources have shown better patency rates relative to unseeded grafts, but a direct comparison between the effectiveness of vessel- and fat-derived cells is challenging as far fewer studies have been done with fat cells [50]. However, an important consideration in choosing an EC source is minimizing trauma to patients during cell harvesting. Harvesting ECs from either vessels or fat is non-trivial, involving surgical removal of tissue. Such procedures are preferably avoided as they expose patients to risks of infection and surgical complication prior to the subsequent
operation to implant the prepared synthetic graft. Consequently, sources of ECs requiring noninvasive or less invasive harvesting methods are being actively explored.

1.1.5 Vascular graft assessment modalities

The assessment of natural, synthetic and tissue-engineered vascular grafts and graft materials has been accomplished via their subjection to different treatments followed by examination of a variety of endpoints. These endpoints can be mechanical, chemical or biological in nature. Standardly characterized mechanical parameters include tensile strength, dynamic compliance, burst strength, and suturability. Hemodynamical characteristics such as luminal sheer stress and the impact of the graft on laminar and pulsatile flow profiles are also considered. Assessed biological endpoints include both hemocapatiblity and the gross examination of implanted grafts. Hemocompatibility is concerned with molecular factors associated with thrombogenicity including hemolysis, platelet activation, and analysis of coagulation cascade activation at various stages of the intrinsic and extrinsic pathways. Implanted grafts and vascular tissue adjacent to anastomosis sites are often grossly examined for patency, thrombus deposition, and intimal hyperplasia in addition to histological examination for inflammatory, endothelial and smooth muscle cells.
Graft performance can be assessed in vivo, ex vivo or in vitro, with each environment uniquely enabling assessment of different endpoints. The advantages and disadvantages of each test environment are considered below.

1.1.5.1 In vivo

In vivo testing represents the “gold standard” of vascular grafts and other biomedical devices as performance is assessed under conditions in which they will ultimately be used. Surgically implanted vascular grafts thus yield the most clinically relevant data. In vivo evaluation typically begins in small animal models such as rats due the lower cost and ease of working with smaller animals. If the vascular constructs show promise, the size of the vascular constructs is scaled up and larger animals such as swine and primates are used due to their cardiovascular systems being similar to humans both anatomically and physiologically. This includes analogous blood vessel sizes, hemodynamics, coagulation systems and metabolism that all better mimic the conditions experienced by grafts implanted in humans and thus increase the clinical pertinence of the results. Encouraging data stemming from these studies then leads to the ultimate test of graft performance – implantation into humans in clinical trials.

In vivo testing offers the advantages of long-term testing in the context of physiologically relevant cardiovascular systems, offering the best prediction of real-
world performance. Only this type of testing can offer insight into interplay between
the graft and adjacent native vasculature that develops over time. For example, stenosis
caused by intimal hyperplasia occurs over months of non-native hemodynamics
stemming from geometrical and compliance mismatches between the graft and native
tissue and thus cannot be studied ex vivo or in vitro. Other types of tissue remodeling
and the long-term effects drug-eluting, in situ-seeded (cell-capturing) and biodegradable
grafts are only observable using in vivo models. The primary disadvantages of in vivo
work are (1) the high cost and labor intensity involved with longstanding animal
husbandry and (2) the consequently low-throughput testing of grafts.

1.1.5.2 Ex vivo

Ex vivo testing is typically accomplished using a shunt that directs blood
through an extracorporeal vascular graft and then back into the circulation. This is done
in large animals such as sheep, dogs, baboons and pigs as smaller animals do not have a
sufficient blood supply or flow rate to support this methodology. Such assessment
is necessarily short-term (hours) as it must be completed before the end of the surgical
procedure, distinguishing it from in vivo studies that offer evaluation of long-term
endpoints. Additionally the graft is situated outside the body instead of as an
anastomosis, the effects of the graft on adjacent native vessels and vice versa are not able
to be explored. Ex vivo testing is thus much more limited and principally useful for
endpoints such as endothelial cell retention, adhesion of activated platelets and leukocytes and thrombus formation.

The advantages of this testing over in vivo testing are higher throughput testing of grafts and lower costs of animal husbandry due to the relatively short experimental duration. However, any animal work is still relatively labor-intensive, expensive and low throughput compared with in vitro experiments.

1.1.5.3 In vitro

In vitro testing offers tremendous potential as a high-throughput, cost-effective alternative to animal work and is consequently preferred as an initial screening platform for promising new vascular graft types. While its endpoints are more limited due to the graft not being directly tested in a live cardiovascular system, well-defined in vitro systems offer their own set of benefits such as facile assaying of molecular hemocompatibility markers and the timecourse of thrombus formation via a variety of methods 61-65.

The most compelling in vitro assays are those that best recapitulate the cardiovascular environment in which grafts will eventually be used. Typically, these are mock circulation loops that subject grafts to flowing whole blood similar to the setup of an ex vivo shunt. Popularly utilized set-ups include the Chandler loop, a vertically oriented circular tubing construct that is filled with blood and rotated to induce gravity-
driven blood flow, and that the roller pump closed-loop system, which uses a pump to actively drive the flow of blood \textsuperscript{66,67}. Each system has its own set of pros and cons, but both are limited in their departure from native hemodynamics experienced in vivo and ex vivo. Substantially more complex set-ups designed to recapitulate in vivo human circulatory hemodynamics have been developed using multiple computer-controlled reservoirs and pumps, but to-date these have predominantly been reserved for testing ventricular assist devices \textsuperscript{68,69}. On the other end of the spectrum are in vitro systems that evaluate grafts under static (non-flowing) conditions, which still provide insight into the hemocompatibility of vascular graft materials outside the context of hemodynamics \textsuperscript{62,63,70,71}. While all of these systems suffer from limitations, they offer great utility as screening tools that could provide sufficient insight to identify promising high-performance grafts ahead of moving to costly and labor-intensive animal models.

1.2 Endothelial progenitor cells (EPCs)

EPCs encompass a spectrum of circulating angiogenic and mature endothelial cells that can be isolated from blood and collectively have the capacity to foster angiogenesis and promote vascular repair \textsuperscript{72-77}. Defining these cells and classifying them into subtypes has been the source of much debate, and there is a standing call from leaders in the field for improved characterization of their biomarkers and functions both in vitro and in vivo. This has been especially challenging due to a spectrum of
differentiation states from progenitor to mature cell along multiple lineages and the potential “programming” of progenitors during in vitro culture, resulting in the study of cells that may not even exist in vivo. The place of LO-EPCs amongst other EPC types given the current understanding of the field is discussed below.

1.2.1 Contemporary knowledge of EPC subclasses

EPCs can be largely classified into the categories of circulating angiogenic cells (CACs) or LO-EPCs (also called endothelial colony forming cells). CACs, widely also known as early-outgrowth EPCs and colony-forming unit ECs, are a mixed population of relatively abundant immune-like cells that form colonies when isolated via culturing buffy coat cells on fibronectin. As their name implies, CACs are thought to promote angiogenesis in vivo by homing to the perivascular space or sites of vascular injury and releasing pro-angiogenic cytokines. CACs predominantly express hematopoietic, monocytic and angiogenic T-cell markers and are thought to be (or at least related to) these cell types. These cells also possess at least some monocytic function as they have ability to phagocytose bacteria. While CACs do secrete angiogenic cytokines and were thought to share some EC surface markers, new evidence suggests that observed EC biomarkers on CACs may be non-cellular artifacts that survived isolation from blood and are present in culture. Some CAC subpopulations may also have the ability to differentiate into mature endothelium without necessarily being committed
to the endothelial lineage. However, it is not clear whether this fraction of CACs mature to ECs in vivo or if it is the result of cellular “reprogramming” by environmental cues such as direct cell-cell contact or transfer of material between cells during culture in vitro following isolation \(^{79,94}\). Having termed CACs as endothelial progenitors has consequently been misleading and generally inappropriate.

LO-EPCs are in contrast a tiny subpopulation of highly proliferative cells that are largely indistinct from mature vascular ECs in terms of known function and biomarkers following culture in vitro \(^{95,96}\). It is this conventional EC behavior, especially anti-thrombogenic and anti-hyperplastic properties, that makes LO-EPCs highly sought after for autologous endothelialization of therapeutic blood-contacting devices such as vascular grafts \(^{55}\). Though these cells are able to be isolated alongside CACs from buffy coat and emerge following long-term (> 2 week) culture, the origin of these cells is unclear as they appear to be clonally distinct from CACs and lack hematopoietic markers. While LO-EPCs may be being derived from maturation of progenitors during co-culture in vitro, an alternative hypothesis is that the cells are in fact not bone marrow-derived but instead shed from endogenous vascular endothelium \(^{97}\). This latter case is also believed to be the case for circulating endothelial cells (CECs), which are mature ECs that do not necessarily possess high proliferation potential.
1.2.2 Sourcing of late-outgrowth EPCs (LO-EPCs)

An immediately available source of host cells is the reservoir of autologous EPCs that can be obtained noninvasively from adult peripheral blood and umbilical cord blood\textsuperscript{97-100}. Autologous LO-EPCs can be rapidly harvested from cord blood, allowing them to be banked in high numbers for future use but necessitating foresight and long-term storage. Obtaining a sufficient number of cord blood EPCs for graft endothelialization would be achieved relatively quickly. However, recent evidence suggests that cord blood LO-EPCs are (1) alloimmunogenic, necessitating immunosuppression even for MHC-matched recipients, and (2) prone to chromosomal instability after long-term culture such as that required prior to endothelializing vascular prostheses, restricting their usefulness and yielding the possibility for the cells to become tumorigenic post-implantation\textsuperscript{101, 102}.

In contrast, autologous peripheral blood LO-EPCs are readily obtained from donors. They also exhibit (1) minimal alloimmunogenicity \textit{in vitro} and \textit{in vivo}, and (2) chromosomal stability after prolonged culture\textsuperscript{103-106}. More importantly, we recently reported that LO-EPCs isolated from the peripheral blood of both CAD patients and healthy volunteers had the same EC-like phenotype when cultured under identical conditions, making these cells viable source for the group with the highest need for synthetic vascular grafts and other vascular devices\textsuperscript{99}. In our recent studies, LO-EPCs
from CAD patients exhibited no signs of being diseased and we have expanded these LO-EPCs repeatedly into healthy and intact endothelium that dramatically improved the patency of small-diameter ePTFE vascular grafts in an athymic rodent model 55.

1.2.3 Clinical significance of LO-EPCs

1.2.3.1 Therapeutic significance

EPCs as a whole have demonstrated therapeutic value as potent stimulators of neoangiogenesis and vascular repair, but the LO-EPC subset of these cells is chiefly useful for endothelialization of vascular grafts and other blood-contacting materials to improve their in vivo performance 76, 96, 103, 107. Current sourcing of ECs involves traumatic surgical intervention prior to another surgery to implant the endothelialized device 50, 108. LO-EPCs offer tremendous potential as an autologous, immediately available, healthy and non-invasively acquired of mature endothelium with the only caveat being their rarity 55, 99. The scarcity of these cells in blood dictates that they be isolated and expanded ex vivo – a procedure that is inefficient, technically challenging and clinically impractical using conventional isolation methods due to the long duration needed to amass sufficient cells.

In vivo studies using grafts endothelialized with EPCs have been encouraging. One group isolated and expanded EPCs ex vivo and then sodded them into ePTFE grafts that were subsequently implanted into rabbit carotid arteries 109. However, the group
isolated EPCs based solely on presence of the CD34 marker and thus a heterogeneous cell population not limited to EPCs was used. Nonetheless, rapid graft endothelialization and a significant inhibition of neointimal hyperplasia were observed. In another investigation, reendothelialization of a catheter-denuded rabbit carotid artery with EPC cells was demonstrated to restore EC-mediated vasodilation \(^{110}\). This is a critical function performed by ECs and a testament to the EC-like phenotype of EPCs and their potential for use in graft endothelialization.

Late-outgrowth EPCs thus have enormous potential as endothelializing cells in prosthetic vascular grafts. They possess the anti-thrombotic and anti-intimal hyperplasia profiles of mature ECs, yet they are an autologous source that is readily and noninterventionally available. Clinical trials with heterogeneous populations of EPCs have been promising and demonstrated improved graft patency, which could almost certainly be further improved by sodding grafts purely with late-outgrowth EPCs.

1.2.3.2 Diagnostic significance

Circulating EPC levels point to the current state of the cardiovascular system and are thus able to serve as an indicator for the risk and progression of vascular disease, cancer and diabetes \(^{81, 111, 112}\). As risk or severity of vascular disease increases, a decrease in EPC levels is observed until an adverse event (plaque rupture, ischemia, stroke, etc.) occurs that sparks mobilization of these cells from marrow to promote injury repair \(^{113}\).
This decrease has also been correlated with a higher Framingham risk score, the gold standard in cardiovascular risk assessment that accounts for numerous risk factors. Interestingly, even in healthy volunteers, the earliest physical sign of atherosclerosis – thickening of the intima and media – is correlated with lower EPC levels and is independent of the Framingham risk score. A recent and excellent review by leaders in the field illustrates this nicely as shown in Figure 1.

Figure 1: A graphical representation of circulating EPC levels as a function of vascular health as conceived by Fadini et al. EPC levels gradually decline during atherosclerotic progression, with levels declining faster and to a greater degree in individuals at higher risk for adverse cardiac events. Adverse events trigger mobilization of cells to repair vascular damage, but this repair mechanism is retarded in higher risk individuals.
EPC levels are also decreased and act as a marker in those with both Type I and Type II diabetes \textsuperscript{112}. A secondary manifestation of diabetes is crippled EPC mobilization, reducing the number of circulating EPCs. EPCs isolated from diabetic patients are also dysfunctional, exhibiting reduced angiogenic capacity and adhesion to activated endothelium in vitro relative to those isolated from healthy subjects \textsuperscript{119,120}. Together these contribute to impaired healing and are thought to contribute diabetic vasculopathy.

Increased EPC (and specifically LO-EPC) levels are observed in the blood of patients with progressive cancers since mobilized EPCs contribute to and result from tumor vascularization \textsuperscript{121,122}. In addition to being indicative of cancer and its progression, EPC counts have also been used to track the efficacy of anti-angiogenic drugs used treat cancer by stifling tumor growth \textsuperscript{111,123}.

While in general EPCs clearly serve as a promising diagnostic marker across a spectrum of disease states, most studies have (had to, in lieu of better definitions) treated EPCs as a lumped parameter instead of delineating between or specifying EPC subclasses \textsuperscript{72,81,103,124}. Differentiating between EPC subtypes would provide clinicians with more detailed information and enable development of more sensitive and specific metrics with which to assess and monitor these and other diseases. It would also bring clarity to the literature which suffers from contradictory reports; for example, while
generic EPC levels are lowered during atherosclerotic progression, one group observed elevated levels of specifically LO-EPCs in patients with more advanced vascular disease as determined by conventional adhesion-based assays 125. Unfortunately, addressing the clear need for more discerning assays for EPC subpopulations has been unfeasible to-date owing to muddy phenotypic definitions and a lack of uniquely identifying biomarkers.

1.2.4. Poorly defined biomarkers

The inconsistency of isolation and characterization methods across studies coupled with the heterogeneity of resulting EPC populations has confounded elucidation of defining surface markers and functional properties of EPC subgroups. Additionally confounding is that the surface proteome and biological function of LO-EPCs and other progenitors drift throughout the differentiation that occurs both in blood and during extended culture ex vivo, further confounding characterization of these cells 78. There is also some evidence for these cells to differentiate to and dedifferentiate from the mature EC phenotype 81. Consequently, though they share characteristic EC markers, currently no uniquely identifying surface markers for LO-EPCs (or other EPC types) have been discovered despite tremendous effort from leaders in the field 81, 126-128.
As a whole, EPCs have classically been defined as displaying the multipotency marker CD34 along with at least one marker from mature ECs such as VEGFR-2 (a.k.a. KDR, Flk-1), von Willebrand Factor, VE-cadherin or CD31 (PECAM)\(^{98,129-131}\) (though as stated above some of these markers may be isolation artifacts). Some groups also define EPCs as having another marker indicating origin (e.g. CD45 – marrow) or stemness (e.g. CD133 – more immature). In lieu of more specific markers and delineating between EPC subpopulations, the profile of CD34+/VEGFR-2+ or even CD34+ alone has been used to enumerate circulating EPCs in the clinic \(^{128,132}\).

Fortunately, an excellent recent study did identify a possible new consensus set of LO-EPC specific markers – (CD34+/CD31+/CD146+/AC133-/CD45-) – determined by rare cell flow cytometric analysis that merits further investigation but gives hope \(^{92}\).

However, without a well-defined and persistent surface marker-based definition of LO-EPCs that isn’t largely shared with other circulating cells, the use of affinity-based technologies for detection and isolation of these cells from blood results in impure populations adulterated with undesirable cells such as platelets, mesenchymal stem cells, smooth muscle progenitors, leukocytes and other hematopoietic cells due to antigenic overlap. This can lead to insensitive and inaccurate diagnostics and, in the context of using isolated cells for endothelialization of blood-contacting devices, co-
isolated undesirable cells could potentially promote inflammation, hyperplasia and even plaque formation that precipitate failure of vascular grafts and other implants\textsuperscript{133-135}.

1.2.5 Challenges of contemporary LO-EPC isolation strategies

Adhesion-based isolations are currently the only viable method by which to isolate and expand LO-EPCs\textsuperscript{100,136}. Whole buffy coat is standardly seeded onto collagen- or fibronectin-coated surfaces and LO-EPC colonies emerge as less adherent cells are gently washed away over several weeks. Alternatively, some groups have “pre-sorted” buffy coat cells to plate only CD34+ or CD133+ cells, but this has not improved – and in fact may have impaired – the isolation efficiency LO-EPCs\textsuperscript{126}. This may stem from LO-EPCs not expressing these markers at later stages of differentiation\textsuperscript{137}.

There exist both technical and biological challenges associated with this seemingly simple isolation strategy. From our own extensive experience isolating LO-EPCs, we estimate that only 50-60\% of isolation attempts from both healthy volunteers and patients with vascular disease are successful. Other groups have published similar rates that seem to be independent of donor health, sparking the isolation of LO-EPCs to be termed an “on/off phenomenon” by a prominent review\textsuperscript{78,81,138}. Interestingly, one study showed excellent reproducibility of LO-EPC isolations from any one donor despite an overall isolation rate of 46\% across all donors\textsuperscript{78}. This suggests a difference in
the average number or phenotypical state of LO-EPCs in some donors versus others that significantly reduces the efficiency of adhesion-based isolations.

Unfortunately, the combined scarcity (~3 cells/mL blood) and conventional isolation efficiency of LO-EPCs from peripheral blood currently imposes a prohibitively long (~5-6 week) lead time to prepare a sodded coronary artery graft and many other blood-contacting implants. Technology enabling the rapid and efficient affinity capture of LO-EPCs from peripheral blood would make the use of LO-EPCs both pragmatic and clinically relevant.

1.3 Aptamers for high-fidelity cell discrimination and purification

1.3.1 Aptamers

Aptamers are single-stranded nucleic acid oligomers that possess monoclonal antibody-like affinity (Kd ~ 10^{-9}-10^{-12} M) and specificity that can be raised against practically any molecule or surface using the well-established directed evolution technique of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) \(^{139-142}\). Aptamer primary structures are designed to have a centralized random region (25-60 bases) flanked by constant regions that enable primer binding for PCR amplification of sequences that survive selection \(^{142,143}\). In solution, these single-stranded oligomers fold into unique tertiary structures which then interact with targets through conventional intermolecular forces of interaction (electrostatics, H-bonding, Van der Waals forces).
Further structural changes typically occur upon interaction with a specific target via adaptive recognition to adopt a conformation providing a more thermodynamically favorable interaction and consequently higher binding affinity \(^{141}\).

Structural analysis of selected aptamers reveals that the contact region made with the target is usually very small (<15 bases) and part of intramolecular secondary structures such as hairpin loops, pseudoknots, bulges, or G-quadruplexes \(^{144,145}\). This small area of contact – typically 300-400 Å, similar to that of antibodies – is the source of the high binding affinities of aptamers. Consequently, post-discovery processing of aptamers typically involves identification of the binding region and subsequent truncation to shorten the length of the aptamer while preserving its binding properties.

Aptamers are also a highly compelling alternative to antibodies. Compared with antibodies, aptamers benefit from (1) superior structural stability and long-term storage capacity, (2) minimal batch-to-batch variability, (3) a high degree of control over chemical modification, (4) generation without the use of cells or animals, and (5) the ability to be evolved against unknown targets \(^{146}\).

### 1.3.2 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

SELEX is an in vitro selection technique that exploits the principle of “survival of the fittest” to progressively enrich an initially random population of nucleic acids in stronger binders toward a particular target \(^{140,142}\). A random nucleic acid pool of \(~10^{15}\)
unique sequences is initially incubated with a purified target of interest (small molecule, protein, specific cell type, etc.) under weak selection pressure to positively select for binders. Binders are partitioned from non-binders, eluted from binding targets and then amplified to generate a new pool of oligos with enriched affinity for the target. A counter selection step to improve specificity by removing sequences that bind similar, undesired targets can then be performed by incubating the new pool with these targets, retaining non-binders and then performing another round of positive selection using the subtracted pool. This procedure is conventionally repeated while increasing the positive selection pressure at later rounds to retain and populate the pool with only the strongest binders until no further enrichment is detected in binding assays.

1.3.2.1 Subtractive whole-cell SELEX and biomarker discovery

An extension of this technique called subtractive whole-cell SELEX (Figure 2) permits aptamers to be evolved to specifically bind “complex” targets such as cells, which have multiple, potentially unidentified or unknowingly unique epitopes in their membrane components 147-149. This technique has been used to effectively discover aptamers that can differentiate between very similar cell types with high fidelity, making it a simple yet powerful tool to isolate a population of target cells without explicit knowledge of discriminating surface features 150-153.
Whole-cell SELEX has been most popularly applied to produce highly selective aptamers that discriminate between cancerous and healthy phenotypes of the same cell type for detection of circulating tumor cells. It has also been used to select for a variety of other cell types including stem cells, vascular endothelial cells and porcine EPCs (truly CACs, not LO-EPCs) \(^{154}\). This power and proven history of this technique make it particularly well suited for the discovery of discriminating affinity ligands specific to LO-EPCs.

Critically, both DNA- and RNA-based aptamers generated by whole-cell SELEX are unstable in vivo due to their susceptibility to degradation by nucleases present in
serum and blood, which can destroy these oligonucleotides within minutes. The greatest bloodborne threats to RNA-based aptamers are RNases A and I, which are relatively minor in abundance compared to RNase H but are able to degrade non-heteroduplexed (i.e. single-stranded) RNA. Nuclease resistance can be conferred to aptamers either (1) directly throughout the selection via the use of non-canonical nucleotides substituted at the 2’ position with fluoro, amino or methoxy groups, or (2) indirectly post-selection via chemical modifications to parts of the aptamer empirically determined to be non-critical for binding the target ligand. As the identities of the aptamer targets are typically unknown in cell-SELEX and purified aptamer target protein is frequently needed for the latter method, it is usually unfeasible for cell-binding aptamers. Consequently, the easier and more economical former method is almost universally used to generate nuclease-resistant RNA (nrRNA) cell-binding aptamers. Non-canonical DNA is contraindicated for in vivo use due to the potential for nucleotides to be recycled into genomic DNA, which can cause mutations that lead to cancer.

Cell-SELEX also provides a facile avenue for biomarker discovery by identifying the unknown cell membrane targets to which aptamers bind. In this technique, aptamer-target complexes are isolated from cell lysate, the target is sequenced via mass spectrometry and the identity of the protein, if known, is determined by analysis using
proteome databanks. The method is especially powerful for revealing novel biomarkers that uniquely identify a specific cell type within heterogeneous population of very similar cells since aptamers against shared biomarkers are subtracted during negative selection steps in cell-SELEX.

1.3.2.2 Efficient aptamer candidate screening via High-Throughput Sequencing (HTS)

As its name implies, high-throughput sequencing (HTS) represents a collection of new technologies that can deliver over a million-fold more reads than traditional methods. This is incredibly impactful when screening enormous random libraries – such as those generated through in vitro selections, including SELEX – for rare yet important sequences such as aptamer candidates.

Following an aptamer selection, a pool is enriched in high affinity target binders but typically still dominated by low-affinity or non-specific binders. Specific aptamer candidate sequences are conventionally identified using traditional sequencing methods, grouping aptamers into families based on consensus sequences and then chemically synthesizing these candidates and characterizing their binding properties. The disadvantage of this method is that up to 20 rounds of selection may be required to sufficiently enrich the pool for effective candidate discovery due to the relatively small sample size that is sequenced.
In contrast, HTS has been utilized to identify aptamer candidates from even whole cell selections in as few as 5 rounds, significantly reducing the time and cost to discover aptamers. The major caveat to HTS is the non-trivial analysis and interpretation of the enormous generated data set, especially given the lack of openly available software to process this data. Many groups have thus been forced to develop and implement algorithms in-house to efficiently sift through HTS data for aptamer candidates.

1.3.3 Comparison to peptides

Antibody fragments (peptides) can also be produced with high affinity and selectivity for targets such as cells using directed evolution techniques similar to that employed for aptamer generation. Multiple “display” platforms exist to generate peptides in which naturally or synthetically derived genomic diversity is translated into an enormous library of antibody fragments and “displayed” to targets (or non-targets) under appropriate selection pressure. Selected binders (or non-binders) are retained and amplified with or without further diversification of the selected peptides and displayed again during the next round of selection. The most notable of these techniques are phage display, ribosome display, mRNA display and yeast display, all of which have been successfully used to produce high affinity ligands to various targets including whole cells. Ribosome and mRNA display technologies are the most similar to SELEX
as the procedures are entirely acellular, avoiding the undesirable transformation of bacteria and yeast, and SELEX offers no clear advantage or disadvantage over these selection strategies in terms of ease or execution.

Moreover, few advantages exist for using nucleic acid-based affinity molecules over their short peptide-based counterparts. The disadvantages of antibodies compared to aptamers chiefly result from the need to generate whole antibodies within cells and the fragile nature of folded proteins. Consequently, short peptides that can be generated acellularly and whose binding properties are not dependent on elaborately folded structures do not suffer from the same shortcomings and offer a viable alternative to nucleic acid aptamers. However, one powerful advantage unique to aptamers is the ability to regulate their function with matched antidotes (discussed below) – greatly increasing their utility in some applications.

In principle both aptamers and peptides should be equally effective at generating cell-specific ligands through a subtractive directed evolution technique like whole cell SELEX. However, the literature on whole cell-based applications of SELEX was by and far more extensive compared to other in vitro selection techniques. SELEX had been repeatedly demonstrated to successfully generate aptamers highly selective for particular cells but not similar phenotypes. Few studies have applied display technologies in the same regard, though notably one group has used phage display to
generate peptides that bind LO-EPC, albeit non-specifically and with unknown utility

1.3.4 Antidote control of aptamer activity

Perhaps the most compelling reason to use aptamers over an alternative affinity technology is their exclusive ability to be modulated by antidotes. Two primary classes of antidotes exist: (1) matched antidotes that are specific for a particular aptamer based on its sequence and (2) universal antidotes which, as their name suggests, can control aptamer activity in a sequence-independent manner.

Matched antidotes are oligomers that capitalize on Watson-Crick base pairing to stably and selectively neutralize an aptamer comprised at least partially of the complementary sequence. Aptamer affinity for its target, and therefore functionality, is developed through its folding into a unique tertiary structure built on favorable intramolecular base pairing. Introduction of an antidote with which base pairing is more thermodynamically favorable than maintenance of its internal structure disrupts aptamer structure and thus function by essentially unfolding the aptamer. Antidotes 15-20 bases in length have historically been sufficient to achieve this effect, with longer antidotes being more specific and more stable. A library of antidotes is usually generated based on complementarity and then screened to identify the best performing (i.e. most potent inhibitor of aptamer function) sequences, and antidotes that have
targeted predicted loop structures have routinely performed very well. Modified RNA antidotes (2’OMe, 2’F, etc.) are nuclease resistant and more potent than their DNA counterparts, making them better suited for in vivo use. The facile development of highly selective antidotes based on the rules of Watson-Crick basepairing thus offers an elegant and powerful solution for modulating aptamer activity. In fact, antidotes paired with therapeutic anticoagulant aptamers are a powerful and clinically successful combination, paving the way for other engineered regulatable aptamer-based drugs.\textsuperscript{178-180}

Universal antidotes offer a powerful, non-specific alternative to matched antidotes. These antidotes are molecules that exhibit a strong positive charge and function by non-discriminately binding nucleic acids, including aptamers, based on their complementary negative charge. The most promising universal antidotes to date in terms of non-toxicity and effectiveness are polymers such as PAMAM and CDP that have also been used to package nucleic acids for delivery based on the same binding principle.\textsuperscript{181} These antidotes are a robust tool for global control of antidote activity, such as when multiple aptamers are employed in a single system and selective control of function offered by matched antidotes is not required.

In theory, antidotes work by neutralizing temporarily unbound aptamer in equilibrium its target. If an antidote is not locally present at this time, the aptamer is
maintains its folded structure and is able to rebind its target and continue exerting its effect. A higher concentration of antidote increases the probability that an antidote will be local and ready to neutralize aptamer upon its dissociation, thus more quickly neutralizing the systemic effect exerted by the aptamer.

1.3.5 Multivalent aptamers

Multivalent aptamer constructs can be constructed from monovalent aptamer building blocks and linkers or scaffolds to significantly enhance avidity and consequently bioactivity. The individual affinities of constituent aptamers contribute to an apparent binding affinity (avidity – the aggregate of multiple affinities) for the entire construct by both crosslinking targets, for example on a cell surface, and also by increasing the local concentration of aptamer near binding targets. Binding theory ideally predicts a 20-fold improvement in apparent affinity for bivalent versus monovalent binders. There is diminishing return with greater valency, however, with trimers offering 15-fold improvement over dimers and tetramers offering 13-fold improvement over trimers. Nature has taken advantage of multivalency with antibodies, which often provide better stability over their monovalent aptameric counterparts. However, bivalent aptamer constructs have been observed to have 10-fold higher avidities and a corresponding 10-fold slower off-rate than their monomeric forms, bringing performance in line with antibodies.
Critically, the design of constructs significantly influences the avidity gains potentially bestowed by multivalency. One group systemically built a library of rationally designed bivalent constructs to explore the influence of (1) distance and (2) linker flexibility between individual aptamers. Both of these parameters were observed to significantly influence avidity. An inter-aptamer distance that was just right yielded 10-fold better avidity over the monomer, whereas a shorter distance only increased avidity 2-fold and a longer distance behaved like the monomer. Flexibility was also required for the best avidity gains, as flexible single-stranded linkers between aptamers provided 5-fold better avidity than rigid double stranded DNA linkers owing to improved rotation and stretching. Some groups have used rigid dsDNA linkers to prescribe an orientation to aptamer ligands based on the pitch of the double helix, but this may do more harm than good in handicapping potential performance gains. For example, one study used tetrameric aptamer with a dsDNA linker and optimistically saw only 20-fold better avidity over the monomer, although they did see substantially better bioactivity as nearly 100-fold less aptamer drug was required to exert a biological effect.
1.3.6 Cell purification

1.3.1.1 Affinity-based Purification techniques

A variety of methods exist for selective affinity-based purification of cells using molecular recognition molecules like antibodies, peptides or aptamers that target specific receptors on the cell surface. These techniques can be used for (1) positive selection in which target cells are labeled and directed isolated or (2) negative selection in which undesired cells are labeled and depleted from the population to yield an impure population enriched in desired cells. Magnetic-assisted cell sorting (MACS) utilizes affinity ligand-coated ferromagnetic beads to positively or negatively select for cells via partitioning with a magnet, usually with good efficiency (>85% recovery)\textsuperscript{188}. The technique is also well-suited for isolation of rare cells such as circulating tumor cells (CTCs), fetal cells, stem cells, sperm, virus-infected cells and bacteria\textsuperscript{189}. Fluorescence-activated cell sorting (FACS) is one of the most popular techniques to purify cells relying on fluorescent affinity molecule-stained cells and flow cytometry to collect purified or depleted cell populations by gating based on size, granularity and fluorescence. However, FACS is not well-suited for capture of rare cells, including specifically LO-EPCs, which in one study needed to be isolated using magnetic beads since they did not survive FACS\textsuperscript{92}. Lower throughput methods such as microfluidics are best suited for capturing low-abundance cells such as CTCs or LO-EPCs with high efficiency\textsuperscript{189, 190}. 
1.3.1.2 Clinical applications

Hematopoietic stem cell transplantation is the most well-established cell therapies and is indicated for treatment of a wide range of diseases, with over 30,000 autologous transplants and 15,000 allogenic transplants performed annually\textsuperscript{191}. The majority of these transplants are used to repopulate the immune systems of patients that have undergone chemotherapy to treat blood cancer. Depletion of T-cells from allogenic donors can be important for mitigating graft-versus-host disease. This can be done using affinity-based cell purification to deplete the graft of T-cells (and sometimes B-cells) either by specifically removing the T-cells based on specific sets of markers or by purifying the CD34+ stem cells that are predominantly responsible for engraftment of destroyed marrow\textsuperscript{192,193}. Therapeutic infusions of CD34+ cells have also resulted in remission of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis and treatment of genetic diseases such as sickle cell anemia and severe-combined immunodeficiency disorder\textsuperscript{191,194}. CD34+ cells are also promising for revascularization of ischemic tissues, possibly due to their content of CACs and LO-EPCs as discussed above.

Adoptive T-cell therapy is another cell therapy strategy relying on purification of T-cells. This strategy relies on isolation of T-cells and tumor infiltrating lymphocytes which are then engineered to recognize certain antigens, expanded in vitro and then
infused into patients. This powerful technique can has been shown to treat cancers and viral disease 195-198.

Genome editing and other technologies that enable precise manipulation of cell behavior are also opening the doors for emerging cell-based therapeutics. In turn, the need for affinity-based purification of newly useful cell types will continue to grow into the foreseeable future 199.

1.4 Significance of this work

1.4.1 Novel metric for evaluating vascular graft performance

An explosion of new prosthetic graft technologies is aimed at filling the tremendous and growing unmet need for readily available high-performance small-diameter vascular grafts is occurring, and these approaches will require comprehensive validation prior being implemented in the clinic. In vitro assays offer cost-effective and fast initial screening methods for the most encouraging graft candidates ahead of ex vivo and in vivo testing. Since most in vitro assays are specialized to assess particular biocompatibility metrics, a variety of assays would best predict graft performance. There is thus a strong impetus for continued development and refinement of in vitro methods concomitant with increasing library of graft types requiring evaluation.
1.4.2 Aptamers selective toward LO-EPCs

1.4.2.1 Therapeutic utility

A particularly great and immediate need exists for high-performance small-diameter vascular grafts, with over 20% (100,000) coronary artery disease (CAD) patients lacking suitable autologous vessels for coronary revascularization each year and this fraction increasing annually. While endothelialized small-diameter prosthetic grafts can match the patency rates of some autologous vessels and are thus a viable alternative, using LO-EPCs for this approach is not yet clinically pragmatic due to the long duration (~5-6 weeks) required to prepare a graft of typical size (4 mm ID, 10 cm long). This long post-harvest waiting period is strongly associated with increased morbidity and mortality for bypass patients (Figure 3). The utility of LO-EPCs for generating autologous endothelium on other blood-contacting devices such as stents, tissue-engineered blood vessels and heart valves is restricted in the same regard.

Aptamers that specifically capture LO-EPCs would permit rapid amassment of these cells via more efficient isolation methods, making their use for endothelialization clinically practical (Figure 3). Conventional adhesion-based isolation methods are fickle, inefficient and slow, with many LO-EPC-seeking groups having significant trouble isolating these cells from a typical volume (~50 mL) of collected whole blood. Affinity-based isolation methods also suffer from utilizing a small volume of blood
Figure 3: Influence of initial number of isolated late-EPCs on (1) duration to achieve full graft coverage and (2) risk of an interim cardiac event given this duration. A higher amount of starting cells greatly reduces the risk to waiting CABG patients. This work may significantly reduce this risk by reducing the lead time for a suitable coronary bypass graft.

and rely on markers shared with undesirable cells, typically resulting in impure populations. The selectivity granted by LO-EPC aptamers would greatly improve the efficiency of affinity-based isolations and afford much purer LO-EPC populations.

However, the greatest impact would come from directly filtering circulating patient blood via an ex vivo shunt through a column containing LO-EPC aptamers. It is hypothesized that specifically capturing LO-EPCs from the entire blood volume (~5000 mL) in this manner would allow initial isolation of 150-fold more LO-EPCs than currently possible, especially with the aid of EPC-mobilizing drugs to significantly
bolster the count of these cells in the circulation \(^{90, 116, 208, 209}\). Such a strategy would reduce the wait time to prepare a typical graft from \(~6\) to \(~2.5\) weeks (Figure 3), substantially reducing risks to waiting bypass and greatly accelerating preparation of other urgently needed endothelialized biomedical implants.

1.4.3.1 Diagnostic utility

Aptamers toward LO-EPCs and other EPC varieties offer an excellent solution for better defining and detecting these cells. Discovery of biomarkers unique to each EPC subset would place them in a clearer context and enable their highly accurate and sensitive detection for reliable and robust clinical diagnostics that will both provide new insight into these diseases and aid in their monitoring.

1.4.3 Antidote-reversible aptamer stains for purification of natively functional cells

Lingering antibody stains traditionally used for affinity-based cell purification can be troublesome in downstream clinical and basic research applications. Antibodies are highly stable and selective labels but their binding is essentially irreversible, resulting in their persistence after being used to isolate cells. This is problematic for cell therapy, as the presence of foreign antibodies on donor cells can result in their destruction by the host immune system following administration. Antibodies also often serve as antagonists that neutralize the function of their target receptors, which can compromise native cell function and potentially their therapeutic effect. Basic research
on cells isolated using a receptor of experimental focus can also be hampered by residual stain by the same rationale.

Aptamer-based stains also enable selective cell purification, but the ability to neutralize their function with antidotes potentially enables removal of these stains after isolation of target cells. This has not been demonstrated but is highly desirable as motivated by the disadvantages noted above for antibodies. Like antibodies, some aptamers neutralize target receptors and block their native function. Successful post-purification removal of aptamer with antidote would theoretically rescue compromised function, yielding purified cells with native behavior. Such a technique would greatly increasing the utility of cells isolated for therapy and basic research. Even if this strategy did not completely remove all of the aptamer stain, cell function should be greatly improved. Their non-immunogenicity also makes and lingering aptamer stain a non-issue for therapeutic use of cells.
2. Coagulation-induced resistance to fluid flow in small-diameter vascular grafts and graft mimics measured by purging pressure

The text and figures of Chapter 2 were previously published in the November 2013 issue of the Journal of Biomedical Materials Research Part B: Applied Biomaterials. The full citation is:


John Wiley and Sons does not require permission for authors to reuse their own articles, but an optional formal license can be obtained and is included in Appendix A.

2.1 Introduction

It is estimated that in 20% of the 500,000 coronary artery bypass grafting (CABG) surgeries performed annually in the United States, patients lack suitable autologous vessels for autografting due to the progression of atherosclerotic disease or due to prior surgeries. This fraction is expected to grow as both the incidence of repeat interventions and mean life spans of people increase. Unfortunately, no current synthetic small-diameter grafts are suitable for CABG because they fail with high
frequency due to thrombosis and intimal hyperplasia—both of which give rise to resistance to blood flow. In general, the level of resistance to blood flow that leads to ischemic effects occurs well before complete vessel blockage 211.

In vitro methods used to study thrombus formation in actual synthetic grafts have focused on endpoints such as graft patency, lumenal coverage of adherent thrombus, amount and morphology of adhered platelets or amount of particular biomolecular species generated by the coagulation cascade 39,212–216. To our knowledge, no in vitro studies have measured the relationship between the extent of coagulation in the vessel lumen and the resultant resistance to flow.

Two primary physical changes occur within the vessel lumen during thrombus formation: cross-sectional area available for blood flow decreases and blood viscosity increases as the blood coagulates 217–220, both of which lead to increased resistance to blood flow. According to the electrical circuit analogy for Poiseuille’s law, which assumes the ideal case of laminar flow of a Newtonian fluid through a tube, resistance to flow is given by the following equation:

\[
R = \frac{8\mu L}{A_{\text{available}}^2}
\]

(1)

where \( R \) is resistance to flow, \( \mu \) is fluid viscosity, \( A_{\text{available}} \) is the cross-sectional area available for flow (neglecting potential porous flow through clots for simplicity), and \( L \) is the length of the tube contacting the fluid 221,222. The resistance to flow in blood
vessels is more commonly described as being patent (lumen unblocked) or occluded (lumen partially to completely blocked). Rearrangement of Eq. (1) expresses the relationship in terms of the cross-sectional area of the unblocked patent vessel \( (A_{\text{patent}}) \) and the cross-sectional area of the lumen occluded by thrombus \( (A_{\text{occluded}}) \), where the percent vessel occlusion is given by \( 100 \times \left( \frac{A_{\text{occluded}}}{A_{\text{patent}}} \right) \).

\[
R = \frac{8\mu L}{A_{\text{patent}} \left( 1 - \frac{A_{\text{occluded}}}{A_{\text{patent}}} \right)^2}
\]

To a first approximation, resistance to blood flow should be linearly proportional to blood viscosity and inversely proportional to the square of the cross-sectional area still available for flow \(^{223,224}\). However, thrombus formation in a vascular graft imposes a number of non-idealities: (1) blood is non-Newtonian; (2) coagulating blood transitions from a watery liquid to a viscous fluid and ultimately to a stiffening viscoelastic clot; and (3) adherent thrombus introduces a non-uniform cross-sectional area along the length of a graft.

In the current study, a closed pressure system connected to small-diameter tubes (either 3 mm inner diameter (ID) synthetic vascular grafts or graft mimics) was used to measure the coagulation-induced buildup upstream pressure within the graft lumen (Figure 4). This build-up of “purging pressure” is an indicator of flow resistance as shown in Eq. (3), where \( Q \) is the average rate of fluid flow and \( R \) is the average
Figure 4: Method used to make measurements with the devised system. (A) Air pressure was monitored proximal to tested vascular grafts via two types of pressure sensors to provide sensitive measurements over a larger dynamic range. Tygon grafts were attached to the vertically oriented system and solutions were drawn into mock grafts using a syringe pump. Alternatively, ePTFE grafts were manually loaded with solution and attached to the system (Figure 5). After allowing the solution to cure for an appropriate duration, graft contents were purged at a constant flow rate. (B) Resistance to flow while purging results in compression of air proximal to the graft until a maximum pressure is reached. This maximum pressure served as the primary end point for pressure measurements.

Resistance between sampled points of pressure upstream (P_{purging}) and downstream (atmospheric pressure, P_{atm}) of a lumenal occlusion.

\[
P_{\text{purging}} = Q \frac{8 \mu L}{A_{\text{available}}} - P_{\text{atm}} = QR - P_{\text{atm}}
\]

Grafts were loaded with fluid and allowed to cure for set times. The cured graft contents were then compressed at a constant rate until the graft contents were purged. The resultant buildup of pressure proximal to loaded grafts was measured continuously by pressure transducers. The greater the extent of coagulation in the graft lumen the
higher was the pressure needed to purge the coagulum from the graft. The pressure just prior to purging (maximum purging pressure) served as an indicator of resistance to flow and was the primary endpoint for experiments.

The pressure system response was validated by purging a series of solutions of known viscosity from Tygon tubes with well-defined cross-sectional areas available for flow as economical and non-permeable small-diameter conduits for initial system validation. The system was then applied to purging cured solutions of coagulating fibrin glue, platelet-rich plasma (PRP) and whole blood from small-diameter Tygon tubes and permeable ePTFE vascular grafts. To our knowledge, this work is the first demonstration of using upstream pressure buildup to quantify the physical interaction between the lumen of a vascular graft and a coagulating clot.

2.2 Materials and methods

All methods were performed at room temperature (approximately 20°C) unless otherwise noted.

2.2.1 Materials

Tygon laboratory tubing with ID of 3 mm and wall thickness of 1 mm (Saint-Gobain Performance Plastics, Akron, OH) was used as a mock graft material. The vascular grafts were tubes of expanded polytetrafluoroethylene (ePTFE) with 2.9 mm ID and 0.21 mm wall thickness (International Polymer Engineering, Tempe, AZ). Tygon
and ePTFE tubes cut to 4 cm in length were sonicated in distilled water for 10 min, absolute ethanol for 10 min, water again for 10 min and then dried with nitrogen gas and in a 50°C oven for 1 h. The tubes were used within 2 h of cleaning.

Mixtures of pure glycerol (Sigma Aldrich, St. Louis, MO) and distilled water were used to generate 0, 50, 80, 90, 95, and 100% (v/v) glycerol solutions. This set of glycerin solutions was used for all experiments to minimize experimental variability.

Fibrin glue solutions were prepared by combining solutions of 12 mg/mL bovine fibrinogen (Sigma) and 0.25 U/mL bovine thrombin (Sigma), each in Dulbecco’s phosphate-buffered saline (DPBS, pH 7.4, Gibco) supplemented with 1% (w/v) bovine serum albumin (Sigma).

2.2.2 Fractional occlusion of Tygon tubes

Bondable (etched) PTFE sheets (Small Parts, Logansport, IN) of thickness 0.03 inch were cut with a VLS6.60 laser system (Universal Laser, Scottsdale, AZ) into a series of annuli with an outer diameter of 7 mm and IDs of 2.683, 2.324, 1.897, 1.439, 0.949, 0.671, and 0.424 mm. After cutting out the annuli, vulcanized PTFE debris was gently removed with a hobby knife (X-ACTO, Columbus, OH) and digital calipers (Mitutoyo, Aurora, IL) were used to confirm IDs.

The annuli were fixed to the exit orifice of the 3 mm ID Tygon tubes, thus decreasing the cross-sectional area available for flow to 20, 40, 60, 77, 90, and 95% of its
original value. A thin film of industrial superglue (Elmens Products, Columbus, OH) was used to center the annuli in the tube opening, followed by two additional applications of superglue at the annulus-tube interface.

2.2.3 Blood collection and plasma isolation

Approximately 150 mL of peripheral blood was drawn by venous puncture into K2 ethylenediaminetetraacetic acid tubes (Beckton Dickinson and Company (BD), Franklin Lakes, NJ) from a single healthy volunteer according to protocols approved by the Duke University institutional review board. To minimize clotting time (CT) variability, collected blood was pooled into a single container and then aliquoted into three 50 mL centrifuge tubes. Two of the blood-containing tubes were centrifuged at 205g for 20 min at 25°C to generate PRP. The partitioned top layers of PRP were pipetted off the top and combined into a single 50 mL centrifuge tube. Both whole blood and PRP were stored at room temperature and used within 36 h of isolation.

2.2.4 Fluid loading into ePTFE vascular grafts

The porosity of ePTFE allowed coagulating solutions to weep through the tubes, necessitating a particular procedure to load the solutions (Figure 5). Cleaned ePTFE tubes were pulled over the hose barbs (3.17 mm maximum diameter) of male luer-lock tubing adaptors (Harvard Apparatus, Holliston, MA) to form tight seals while leaving approximately 250 μL tube volume beyond the barbs. Maintaining the tube/adapters in
Figure 5: Method for loading clotting solutions into ePTFE vascular grafts. Grafts connected to male luer-lock tubing adaptors were oriented horizontally and then loaded with clotting solutions via a needled syringe. The distal end of the graft then was crimped with surgical ligating clips to prevent leakage upon orienting the graft vertically. After burping trapped air bubbles, clotting solutions were allowed to cure in a vertical orientation for desired durations.

a horizontal orientation, clotting solutions were slowly injected into the tubes via syringes with the needles extending through the adaptors and just beyond the hose barbs. While still horizontally oriented, each tube end was crimped with a surgical ligating clip (Teleflex Medical, Research Triangle Park, NC) just beyond the location of the blood. Loaded tubes were then vertically oriented and “burped” to remove air
bubbles by gently compressing the tubes along their lengths with gloved fingers. Care was taken not to alter the tube cylindrical geometry during this process. Tubes remained in a vertical orientation while clotting solutions cured by coupling the loaded tube/adaptor to a suspended luer-lock syringe.

2.2.5 Fibrin CTs

Fibrin glue CTs were determined in polypropylene 96-well plates (BD). Fibrin glue solutions were prepared as described above to have a final fibrinogen and thrombin concentrations of 6 mg/mL and 0.125 U/mL, respectively. These solutions were then immediately transferred to the well plates and allowed to cure for set durations. The CT was defined as the time at which a fibrin strand could be drawn from the solution with a pipette tip. A rough CT was determined first by testing solutions at 1 min curing intervals. A more precise CT was then determined by using shorter curing intervals centered around the approximate CT. This process was iterated until the CT was determined within 10 s.

2.2.6 PRP and blood CTs

Many factors (blood-contacting surface type, surface area to-volume ratio, physical orientation, level of air exposure, etc.) impact the CT of blood and PRP. This required that blood and PRP CTs were measured using the same clotting conditions used in the pressure experiments. Blood and PRP were reactivated with 0.1 M CaCl2 at a
1:10 ratio to blood or PRP. CTs in Tygon tubes were determined by drawing approximately 200 μL reactivated blood or PRP into cleaned tubes attached to 5 mL luer-lock syringes (BD). After blood or PRP were allowed to cure for various durations, tubes were then purged and the contents were examined for evidence of clotting. The blood CT was defined as the shortest curing duration that generated a thrombus. A rough CT were determined first by testing at 10 min curing intervals, followed by more precise CTs determined using shorter curing intervals centered around the rough CT. This process was iterated until the CT was determined within 1 min.

2.2.7 Rheology of glycerin solutions, fibrin glue and blood

    Rheological measurements were performed with a cone and-plate Brookfield DV-III Rheometer outfitted with a LV spring and a CP40 spindle (Brookfield Engineering Laboratories, Stoughton, MA). The empty rheometer was zeroed with an idle torque reading of <1%, and the gap between the cone and plate was established. Volumes of 0.5 mL glycerin solution, fibrin glue, or reactivated blood were loaded, and the viscosity was measured over time using a low shear rate of 2.25 s⁻¹, which was chosen to minimize shear induced activation of blood. As blood approached its CT, the torque on the spring rapidly increased until the torque limit was reached and additional data could not be gathered. RHEOCALC software (Brookfield) was used to record and process raw data into values of viscosity.
2.2.8 Spectroscopic characterization of fibrin glue clotting reaction

Fibrin glue solutions were prepared as described above to have a final fibrinogen concentration of 6 mg/mL and final thrombin concentrations of 0.25, 0.125, 0.0625, and 0.03125 U/mL. These components were mixed, immediately transferred to polypropylene 96-well plates (BD), and absorbances were monitored over time at 405 nm in a μQuant microplate reader (BioTek, Winooski, VT). Absorbance data were baseline corrected, and the time axis was offset to correct for differences between experimental runs in the initiation of clotting and the beginning of data acquisition.

2.2.9 Pressure measurement system

Figure 4(A) shows the pressure measurement system consisting of a vertically oriented luer-lock 5 mL syringe secured in a programmable syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA), and connected to a 3-way T-fitting. One T-fitting connected the syringe to the Tygon tubing or ePTFE grafts. The other two T-fittings connected separately via pressure lines (6 cm lengths of 0.5mm ID low gas permeability PharMed BPT tubing, Saint-Gobain Performance Plastics) to a low-sensitivity/high-resolution pressure transducer (part MPXC2011DT1; FreeScale Semiconductor, Tempe, AZ) and a high-sensitivity/low-resolution pressure transducer (part MPX5100GP, FreeScale Semiconductor). Both transducers were supplied with 5 V DC (CPS250 power supply; Tektronix, Beaverton, OR). The output of the high-resolution transducer was
amplified 10003 and band-stop filtered (0.1–100Hz) by a differential amplifier (DAM 50; World Precision Instruments, Sarasota, FL) running in differential mode. Pressure signals were interfaced with a computer via a National Instruments 9215 compact data acquisition card in a Legacy CompactDAQ chassis (National Instruments, Austin, TX) that permitted simultaneous capture of the two independent pressure transducer signals without introducing crosstalk. Data were acquired using LabVIEW 2010 software (National Instruments). All junctions were connected within short Tygon tubes and hermetically sealed with industrial superglue.

The voltage–pressure relationship of the system was determined by assuming that the product of gas pressure and volume in a closed system is conserved according to Boyle’s law 225. The hose barb attached to the Tygon tubes was closed while maintaining an initial ambient pressure of 760 mmHg. Voltage data from the pressure transducers were recorded while compressing the system in increments of 2% of the known starting volume. Given these values of initial volume, initial pressure and compression levels, the pressure at each compression level was computed and plotted against the voltage signals from the transducers. Calibration curves were then generated for each transducer and used to convert experimental raw voltage data to pressure values.
2.2.10 Purging pressure measurements

Prior to making measurements, the syringe plunger was fully depressed to ensure a consistent and minimal dead space volume of the system. For Tygon tubing, cleaned tubes were pulled over the hose barb of the T-fitting such that approximately 220 µL tube volume remained distal to the barb. The distal inlet of each graft was submerged into a glycerin solution, fibrin glue, PRP, or whole blood, and the syringe pump was used to draw up 200 µL of fluid. Coagulating solutions were allowed to cure for specific durations based on previously determined CTs (Figure 4(A)). Data acquisition was initiated 5 s prior to purging grafts to establish a baseline signal. After curing for the appropriate duration, the syringe pump was used to purge grafts at a constant flow rate of 2.4 mL/min. Due to the resistance to flow of the fluids within the grafts, trapped air proximal to the grafts was compressed, resulting in increased systemic pressure while purging (Figure 4(B)). The accumulated pressure was released upon expelling sufficient fluid or coagulating material to create a path through which the compressed air could escape. The maximum purging pressure is the pressure achieved just prior to expelling the contents of the tubes.

Pressure measurements of ePTFE grafts were performed using the same procedure as above with the following modifications. A luer-lock type connector (Cole Parmer) allowed fluid-loaded ePTFE grafts to be screwed directly onto the T-fitting
attached to the syringe. Just prior to reaching the curing duration of the coagulating solution, grafts were attached and the crimped end of each graft was removed to permit purging by cutting with surgical scissors just above the ligation clip. Purging and data acquisition were then carried out as in the protocol for Tygon grafts.

Acquired raw voltage data were baseline-corrected by subtracting the average voltage value recorded over 5 s while the system was idle before purging grafts. Voltages were then converted to pressure values using the calibration curves generated for each transducer. Maximum pressures achieved while purging grafts were identified for both transducers. Pressure values obtained from the amplified higher resolution transducer were more precise and were thus used whenever possible. Above 60 mmHg, pressure values of the lower resolution transducer were used as the higher resolution signal became saturated.

2.2.11 Modeling theoretical flow resistances

Equation (1) was used to compute theoretical flow resistances from experimentally derived values of viscosity, cross-sectional area available for flow, and tube length. To model fibrin glue resistances, curing fibrin glue was assumed to be a Newtonian fluid with a given apparent viscosity at a particular curing time as determined by rheology. Computed resistances were then normalized to the largest calculated value and plotted.
2.2.12 Statistics

Differences between experimental conditions were determined using multivariate analysis of variance (ANOVA). The significance of individual differences was established using the post hoc Tukey’s honestly significant difference test where appropriate. Significance was assumed at p < 0.05.

2.3 Results

2.3.1 Solution viscosity and fractional occlusion pressure measurements

Figure 6 compares the maximum purging pressure accumulated in the Tygon tubing as a function of percent occlusion (area of exit orifice blocked by the annulus) for a range of fluid viscosities to theoretical flow resistance computed for the same conditions using Eq. (2). The maximum purging pressure increased with increased glycerin solution viscosity and with increased percent occlusion. Each fluid of a given viscosity exhibited a similarly shaped nonlinear increase in purging pressure with increased percent occlusion, with this increase being most pronounced for the most viscous fluids. Each viscosity or occlusion level was significantly different from all others (p < 0.0001 for most comparisons), except for the statistically similar viscosity levels of 1 and 8.3 cP (p = 0.96). Theoretical resistances followed similar trends to maximum purging pressures observed experimentally. In both cases, the blockage-induced pressure did not increase substantially until after 60% occlusion.


Figure 6: Experimentally observed maximum purging pressures for a range of fluid viscosities and cross-sectional areas available for flow exhibited similar trends to theoretical flow resistances predicted by the electrical circuit analogy for Poiseuille’s law. (A) Greater values of solution viscosity and fractional occlusion resulted in greater maximum pressures while purging Tygon grafts. Glycerin solutions ranging in viscosity from 1 to 1410 cP were purged from modified Tygon grafts with a range of percent occlusions, with each occlusion level significantly different from all others (p < 0.0001 except 40 vs. 60% at p < 0.05). More viscous solutions also generated significantly maximum higher pressures, with each tested viscosity significantly different from all others (p < 0.0001) except for 1 vs. 8.3 cP, which were statistically similar (p = 0.96). The inset provides a clearer view of the same data for fractional occlusions ≤60%. Data are mean ± standard error of the mean (SEM); n = 3 and small error bars are obscured by data points. (B) Theoretical flow resistances computed for corresponding experimental conditions possessed similar trends to measured data.

2.3.2 Fibrin glue and whole blood pressure measurements

Figure 7 shows the increasing pressure generated while purging fibrin glue, PRP and whole blood samples from Tygon tubes and ePTFE grafts as a function of curing duration. Note that the data in Figure 7 are presented as a fraction of CT that is defined as the duration required for gelation. This normalization was necessary to correct for
Figure 7: Maximum pressures were (1) maintained for longer upon purging nonporous Tygon grafts of fibrin glue or whole blood cured for longer durations (fractions of clotting time (CT) as noted) and (2) highly variable at the same curing duration upon purging blood and PRP from porous ePTFE grafts. Individual pressure traces (representative of multiple trials) generated while purging fibrin glue (A) and whole blood (B) from Tygon reveal that both the maximum pressure and the period for which that pressure persisted increased with greater curing times. While fibrin glue pressure traces were highly reproducible, blood traces were highly variable in Tygon at and just after the clotting time (two traces of 1.0 and 1.25 CT shown to illustrate variability). (C) Purging whole blood and PRP cured for their clotting times (1.0 CT) from ePTFE was even more variable, with purging pressures ranging from near baseline to ~140 mmHg (three traces of each clot type shown to illustrate variability). Purging blood cured in Tygon for 150% of the clotting time (1.5 CT; ~45 min) (B) and in one case PRP cured in ePTFE for 100% of the clotting time (run 3 of 1.0 CT; ~120 min) (C) resulted in maintenance of maximum pressure until data collection stopped (data not shown).

slight variations in the CT for the different fibrin glue preparations (seconds) and more substantial variations in the CT of reactivated whole blood (minutes). Overall, both fibrin glue and whole blood exhibited the same behaviors in nonporous Tygon tubes. The fibrin glue results yielded the most highly consistent curves for the same curing times (Figure 7(A)); however, blood samples in Tygon tubes exhibited greater variability during the build-up of purging pressures for the same curing times (Figure 7(B)).
Consistent results were also achieved with fibrin glue in ePTFE grafts (individual traces not shown). PRP and whole blood cured in ePTFE grafts exhibited the most variable purging pressures, ranging from near baseline values to ~140 mmHg (Figure 7(C)) at gelation.

Fibrin glue samples cured for less than the CT (1.0 CT in Figure 7(A)) were purged spontaneously when the maximum purging pressure was reached; however, the maximum purging pressure plateaued for fibrin glue cured for longer than the CT before the gelled fibrin was ejected in both Tygon and ePTFE (only Tygon shown). The duration of this plateau increased the longer the glue was cured beyond the CT. This phenomenon also occurred with blood and PRP but was only consistently observed well beyond the CT (1.5 CT in Figure 7) in Tygon tubes. Inspection of the purged grafts at longer curing times showed that the outer portions of the coagulum adhered to lumen wall after the center portion of the coagulum was purged.

Figure 8 shows the effect of curing duration on maximum pressures generated upon purging fibrin glue and blood from Tygon tubes, and purging fibrin glue from ePTFE grafts. All three cases exhibited increased maximum purging pressures with increased curing times (p < 0.0001 for all comparisons except p < 0.05 for 25 vs. 50% greater than the CT). The inset in Figure 8 shows that these higher pressures correlated with increased turbidity of curing fibrin glue solutions. Both whole blood and fibrin glue
Figure 8: Higher maximum pressures resulted from purging fibrin glue and reactivated whole blood cured for longer durations from both Tygon and ePTFE grafts. Fibrin glue was allowed to cure for various durations normalized to its clotting time and then purged from both graft types, and same was done with blood in Tygon tubes. Curing of fibrin glue was also monitored spectroscopically (inset). Maximum pressures and solution turbidity (absorbance at 405 nm) both increased with increasing curing durations, with each curing duration significantly different from all others (p < 0.05 for *, **, ***, and ****). However, no significant differences between the type of graft or clotting solution were observed for a given curing duration. Data are mean ± SEM; n = 4.

exhibited significantly higher maximum purging pressures with increasing curing times (p < 0.0001 for most comparisons). However, maximum purging pressures for whole blood were much more variable near the CT (~30 min), which was substantially longer than the CT of fibrin glue in Tygon (~4 min). Consequently, statistically similar pressures were observed for blood and fibrin glue for all curing durations. Much lower
variability (similar to fibrin glue) was produced from purging blood cured for less than or 50% longer than the CT.

The CTs of whole blood and PRP in ePTFE grafts (~120 min) were also much longer than that of fibrin glue in ePTFE grafts (~4 min). Maximum purging pressures produced by the clotting of whole blood and PRP in ePTFE grafts are shown in Figure 9. Purging whole blood and PRP cured until their respective CTs resulted in significantly greater maximum pressures than purging these clotting solutions just after reactivation with calcium (p < 0.0001). High variability was observed in purging blood or PRP at the CT, with maximum purging pressures ranging from near baseline to almost 140 mmHg. As a result, maximum pressure values for purging whole blood and PRP both before and at the CT were statistically similar despite a large number of trials (n = 10).

2.3.3 Rheology of fibrin glue

The apparent viscosity of fibrin glue increased with curing time (Figure 10(A)) and was found to increase more slowly than did the maximum purging pressure over the same range of curing times (Figure 10(B)). While apparent viscosity increased and seemed to approach an asymptotic value from curing times of 90–150% of the CT, substantially higher variability at longer curing durations resulted in statistically similar apparent viscosity values at and above the CT.
Figure 9: Highly variable maximum pressures were generated upon purging blood or PRP from ePTFE grafts at the clotting time. Reactivated blood and PRP were allowed to cure until the clotting time and then purged from ePTFE grafts. The clotting solutions were also purged just after reactivation for comparison. Maximum pressures generated from purging both whole blood and PRP were extremely variable, ranging from baseline pressure levels to nearly 140 mmHg (also shown in Figure 7(C)). Consequently, maximum pressures generated by purging blood and PRP at each during duration were statistically similar (p = 0.71). Despite this variability, maximum pressures generated at the clotting time were significantly higher (p < 0.0001 for *) than those produced just after reactivation of blood or PRP. Data are mean ± SD; n = 10.

2.3.4 Comparison of experimental results to theory

Figure 11 illustrates how experimentally measured maximum purging pressures deviated from theoretical pressures calculated from Eq. (1) for both pure glycerin and fibrin glue. Measured pressures and theoretical resistances both increased with increasing tube occlusion and apparent viscosity, but maximum pressures generated by purging glycerin and fibrin glue were underpredicted and overpredicted, respectively, by theory.
Figure 10: The apparent viscosity of fibrin glue increased and became more variable as it cured up to and beyond its clotting time. (A) Beyond the clotting time, apparent viscosity increased more slowly and seemed to approach a plateau. Due to the high variability in measurements beyond the clotting time (>1.0), all apparent viscosities are statistically similar except for a significantly lower value at a curing time of 0.9 (p < 0.05 for *). Data are mean ± SEM; n = 3. (B) Maximum purging pressures increased faster and were less variable than apparent viscosity for corresponding curing times of fibrin glue (noted for each data point as fractions of clotting time). Statistics of pressure and apparent viscosity data are identical to those presented in Figures 8 and 10(A), respectively, and are omitted here for clarity. Viscosity data are mean ± SEM, n = 3, and pressure data are mean ± SEM, n = 4.
Figure 11: Comparison of experimental and theoretical resistances generated by purging pure glycerin (gray line) and fibrin glue (black line) from Tygon tubes. The diagonal dashed line indicates perfect correlation of tube pressure described by Eq. (1) and the experimentally determined maximum purging pressures. Maximum purging pressure increased with percent tube occlusion and fibrin glue viscosity as theoretically predicted; however, maximum pressures generated when purging pure glycerin from the range of percent occluded tubes (Figure 6) were higher than predicted by theory, whereas maximum pressures generated when purging fibrin with a range of experimentally measured apparent viscosities from tubes (Figure 10(B)) were lower than predicted by theory.

2.4 Discussion

The purging pressure measurement system described here was capable of precisely detecting physiologically relevant changes in solution viscosity or the cross-sectional area available for flow. Fibrin glue and blood coagulation in Tygon and ePTFE grafts were also precisely monitored, with more developed clots resulting in greater
purging pressures and pressures induced by the presence of blood clots being generally more variable than those of fibrin clots.

The range of purging pressures measured in these studies required the simultaneous use of two pressure transducers to expand the dynamic range of the system and to improve low-end sensitivity. As this dual transducer configuration was capable of highly reproducible measurements with generally tight error, experimental variability that was observed in more complex, less well-defined sample measurements such as fibrin glue or blood could be attributed to the intrinsically high variability of these biological solutions and not the pressure system itself.

The response of the pressure measurement system to the range of physiologically relevant fluid viscosities and percent occlusions was reproduced qualitatively by Poiseuille’s law (Eq. (1)). However, regression plots revealed this law to underestimate the measured pressure increases stemming from increased viscosity and percent occlusion (Figures 6(B) and 11; regression plots not shown). We attribute the deviation of our experimental measurements from Poiseuille’s law (which describes laminar flow in a tube of consistent ID) primarily to the abrupt decrease diameter at the terminus of the Tygon tubes. Other deviations from ideal behavior, such as energy losses due to unaccounted for frictional factors, likely also contributed to experimentally observed maximum purging pressures being underpredicted by computed theoretical values.
Similar to the clinical observation that coronary artery blockage is asymptomatic until around 60–70% occlusion, (private communication226) these results show that both maximum purging pressures and computed flow resistances did not become substantial until tube blockages of 60% or higher.

Fibrin glue with its well-defined reaction kinetics was chosen as a blood clot mimic in the initial testing of clotting solutions with the system. These initial tests were performed using Tygon tubing as economical and non-permeable conduits that possess fluorinated surface chemistries similar to ePTFE grafts. Fibrin glue is a buffered solution containing fibrinogen and thrombin that react to generate an a cellular interpenetrating network of fibrin strands 223, 227. Importantly, the particular formulation of fibrin glue used in this study did not produce cross-linked clots as it lacked factor XIII 228, 229. This highly simplified clotting scheme facilitated the systematic exploration of changes in flow resistance resulting from curing a clotting solution for various durations. In contrast, blood clotting occurs in the context of an enormously complex biological milieu involving erythrocytes, platelets and many additional serum protein components in the coagulation cascade that are absent from fibrin glue. In particular, platelets and erythrocytes are incorporated into and strengthen the fibrin clot by directly binding with and being entrapped by fibrin 230, 231. The presence of factor XIII in blood also permits the covalent cross-linking of fibrin chains, reinforcing the clot and increasing its bulk
strength\textsuperscript{232,233}. Note that two distinctly characteristic clot types, platelet-rich “white clots” and fibrin-rich “red clots” (so-named as they readily entrap erythrocytes), preferentially form under arterial and venous flow conditions, respectively. Blood clots in this study were formed under static conditions and thus likely differed from either of these clot types, but blood coagulum still offered a significantly different alternative clotting material to fibrin glue while possessing much more similar character to lumenal clots formed in vivo.

As fibrin glue cures in the graft lumen, fibrin strands thicken, lengthen and intertwine, increasing clot strength and wall adhesion\textsuperscript{223,234,235}. Blood clots are additionally strengthened over time by the incorporation of platelets and cells into the crosslinking fibrin. We believe that these factors gave rise to (1) stronger resistance to flow at longer curing durations and therefore higher maximum purging pressures and (2) the plateauing of the maximum purging pressure for the most coagulated samples (Figures 7 and 8). However, the inset in Figure 8 shows that the turbidity spectra of curing fibrin glue continued to increase linearly even when the maximum purging pressures did not increase with longer curing durations. The fibrin therefore continued to polymerize substantially even though the strength of the coagulum appeared to no longer increase.
Despite substantial physical and biological differences between fibrin glue and blood coagulum, maximum purging pressures generated at corresponding degrees of coagulation were statistically similar in Tygon grafts (Figure 8). The statistically similar data suggest that the fibrin component of the blood coagulum plays a dominant role in the resistance to flow over the probed range of curing times. However, it is possible that the maximum level of system compression was insufficient to elicit observable differences at 50% beyond the CT. Should differences in maximum purging pressures exist, they may become apparent at higher levels of system compression and/or curing times longer than those tested here.

It should be noted that Tygon tubing is not a vascular graft material, fibrin glue coagulum does not form in the same manner as blood clots, and that static testing does not account for the variety of dynamic flow and environmental conditions that one encounters in vivo. However, our data point to substantially different clot types offering similar resistance to flow when obstructing both mimicked and actual vascular graft materials. That said, virtually all in vitro assays used to assess blood-material interactions, such as whole blood clotting time are designed to be simple and reliable measurement schemes that generate comparative results. The current study suggests that the devised system is potentially capable of assessing a wide range of physiologically relevant clotting conditions in vascular graft materials.
Pressure measurements made using blood and PRP proved to be more difficult. Even though the pressure system was capable of precise measurements and precautions were taken to minimize variability, the highly variable results obtained for blood and plasma clotting reveal the notoriously difficult nature of making quantitative measurements with blood and PRP near the CT (Figures 7(B,C) and 9). Under identical testing conditions, the clotting kinetics for a given blood sample were primarily dependent on the degree of blood activation (i.e., extent of coagulation cascade activation; also applies to PRP)\textsuperscript{236,237}. Indeed, the same blood sample was observed to clot nearly four times more slowly in ePTFE (~120 min) than in Tygon (~30 min)—an effect that was corrected for by normalizing experimental curing times to permit direct comparisons between clotting agents with different CTs. However, this effect should not have contributed to the observed variability in pressure data for these clotting solutions as presented data corresponded to testing within the same graft type. Therefore a number of factors could have contributed to the variability of this data: (1) inconsistency in blood handling, which would have activated clotting pathways to different extents, (2) decreased viability of platelets and erythrocytes (for whole blood) as collected blood and PRP aged, causing a drift in the CT, and (3) differential reactivation of blood and PRP due to having added back unequal amounts of calcium. It is also possible that the
carefully determined CT of blood drifted over the course of the experiments, resulting in clots of differing maturity at the same curing time.

The largest such variability was observed for blood and plasma clotting in ePTFE grafts. The increased specimen handling when loading the grafts with blood may have activated PRP or blood to different extents. Increased variability also stemmed from the spatial variation of blood and PRP clotting rates, with the fastest clotting occurring proximally in the graft. The relatively long CT of blood and PRP (around 2 h) may have allowed (1) some fluid to weep out of the porous material over time and (2) the formed elements of the blood to sediment, enriching the distal ends of the grafts in cells/platelets and leaving mostly plasma in the proximal end to result in inhomogeneous clots. Consistent results were achieved for fibrin glue in ePTFE as its relatively fast gelation kinetics circumvented these issues. A method for loading porous grafts and reducing blood partitioning that minimized specimen handling could mitigate variability and allow meaningful systematic studies to be performed in porous graft materials.

As fibrin glue cures and transitions from a fluid to a stiffening viscoelastic gel, the same biophysical phenomena that led to increased bulk coagulum strength also caused the observed increase in apparent viscosity over time toward an asymptotic value (Figure 10(A)). However, despite also approaching a terminal value as clotting proceeded, maximum purging pressures appeared to increase to a greater degree than
apparent viscosity at later curing times (Figure 10(B)). This discrepancy may have been an artifact caused by the shearing of maturing fibrin clots during the rheological measurements, possibly breaking apart growing fibrin strands, and therefore underestimating apparent viscosity measurements at later curing times.

Finally, theoretical resistances to purging fibrin glue computed using the rheologically determined apparent viscosities were substantially higher than were the corresponding maximum purging pressures (Figure 11). One possibility is that theoretical resistances assumed that the clot structure and apparent viscosities were homogenous throughout the clot, but actual fibrin clots were nonuniform and were locally weaker, particularly in the center region, which may have failed preferentially at lower maximum purging pressures.

2.5 Conclusions

The goal of this study was to quantitatively assess the maximum flow-induced pressure upstream of curing blood coagulum within the lumens of synthetic vascular grafts. By measuring pressure accumulation, the devised system was able to provide a quantitative indicator of the flow resistance that developed during the coagulation of fibrin glue, PRP, and whole blood within the lumens of Tygon tubes and ePTFE vascular grafts. The coagulation of fibrin glue and whole blood both showed a maximum purging pressure that increased with curing time. The fibrin glue data, being the best behaved of
the two coagulums, yielded similar results in both nonpermeable Tygon tubes and permeable ePTFE grafts. Whole blood, being more difficult to work with, also performed similarly in the Tygon tubes but both the permeability of the ePTFE grafts and relatively long and variable CTs proved to be problematic for blood and plasma studies. This approach provided insight into how blood flow resistance is influenced by a number of clinically relevant factors, such as the level of vessel occlusion and the physical nature of the resident coagulum. While the described system could only quantify resistance as a lumped parameter, future refinement of this technique may enable the specific clot failure mechanism (i.e., shearing from lumen or rupture) to be elucidated, and thus provide an in vitro predictive test of graft–thrombus interactions.
3. Aptamers for the selective capture of late-outgrowth endothelial progenitor cells

3.1 Introduction

The specific detection and amassment of circulating late-outgrowth endothelial progenitor cells (LO-EPCs) from whole blood is critical in promising diagnostics and therapeutics for vascular disease, cancer and diabetes that afflict millions of people worldwide.\(^7\) However, identifying and accruing these cells is technically challenging as they are exceedingly rare (~3/mL blood) in blood and without uniquely identifying biomarkers or even an accepted consensus set of markers.\(^77, 78, 81, 92, 93, 99, 103, 124\). This subpopulation of EPCs possesses the desirable highly proliferative EC phenotype that emerges as isolated cells differentiate into mature ECs during culture in vitro, presenting an elusive and moving phenotypic target on which to base affinity-based LO-EPC recognition.\(^78, 138\). High affinity ligands enabling selective targeting of these cells would significantly improve the efficiency of contemporary LO-EPC isolations and facilitate the development of clinically impactful EPC-based biotechnologies that are not currently pragmatic due to the aforementioned limitations.

Differential in vitro selection techniques enable discovery of highly selective ligands by iteratively screening large, random libraries of antibodies, peptides or nucleic acids (aptamers) and selecting for those only specific toward desired target(s).\(^139, 142, 172-174, 242-244\). Perhaps the most successful strategy in generating selective ligands for a
particular cell type without a priori knowledge of specific targets is subtractive whole-cell Systematic Evolution of Ligands by Exponential Enrichment (SELEX), which selects for short oligonucleotides known as aptamers that possess antibody-like affinity and specificity $^{140, 143, 146}$. Aptamers that can differentiate, for example, between diseased (e.g. cancer) and healthy cells of the same tissue with exquisite selectivity have been discovered through this technique and been used to robustly detect rare target cells residing within whole blood $^{148, 152, 245, 246}$. Further, elucidating the identities of targets to which these aptamers bind enables facile discovery of potentially unknown and uniquely identifying biomarkers that contribute to the phenotypical definition of the target cells $^{153, 162, 163}$.

The overarching goal of this project was to identify and characterize nuclease-resistant RNA (nrRNA) aptamers selective toward LO-EPCs residing within whole peripheral blood. Identified aptamers would have the potential to (1) significantly improve the efficiency of detecting and isolating these cells and (2) enable discovery of biomarkers unique to LO-EPCs amongst other circulating human cells. Only nuclease resistant aptamers are stable in vivo and thus have utility in capturing cells directly from whole blood – one of the most promising avenues to rapidly amass LO-EPCs for endothelialization – in addition to being useful in less demanding in vitro applications.
Critically, aptamers can only be discovered using LO-EPCs that have been expanded ex vivo but need to be able to bind LO-EPCs present in circulating blood for clinically relevant applications. Meeting this criterion is non-trivial given the phenotypic changes that occur during differentiation in culture. An attempt to “universalize” LO-EPC aptamers was made by subtracting out markers not common to all LO-EPCs via the use of cells (1) from different CAD patient donors and (2) at different passage numbers. LO-EPCs isolated from CAD patients were used as targets since the aptamers would predominantly be used in the diagnosis and treatment of this demographic.

An overview of the proposed work is shown in Figure 12. The initial phase of this study aimed to generate nuclease-resistance RNA (nrRNA; 2′-OMe modified pyrimidines) aptamers that bind both cultured and circulating late-EPCs of CAD patients with high specificity and affinity but not other circulating cells using subtractive whole-cell SELEX. This technique was implemented in attempt to progressively enhance the selectivity toward LO-EPCs of an initially random nrRNA library until no further enrichment in aptamer selectivity was observed by flow cytometry.

Subsequently proposed work sought to characterize the discovered aptamers and evaluate their abilities to capture LO-EPCs. High-throughput sequencing (HTS) was to be performed to aptamer candidates whose binding capacities toward both LO-
Figure 12: An overview of the proposed work. An iterative in vitro selection method called subtractive whole-cell Systematic Evolution of Ligands by Exponential Enrichment (SELEX) was be used to evolve nuclease-resistant RNA (nrRNA)-based aptamers that bind LO-EPCs with high specificity from an initially random nucleic acid library containing $\sim 10^{15}$ unique oligomers without a priori knowledge of the binding targets. nrRNA pools from later selection rounds was then to undergo high-throughput sequencing to identify aptamer candidates with unique sequences. Aptamer candidates were then to be individually tested for their apparent binding strengths to LO-EPCs from various donors and buffy coat cells. The binding targets on LO-EPC surfaces of the most selective aptamers were then to be elucidated by mass spectrometry to potentially reveal novel and uniquely characteristic LO-EPC surface markers. Finally, the utility of the generated aptamers to selectively capture both cultured and circulating (non-cultured) LO-EPCs using aptamer-conjugated magnetic beads was planned to be evaluated.

EPCs and buffy coat cells would have been determined by flow cytometry. The binding targets of aptamer candidates was then going to be determined by performing mass spectrometry on digested aptamer-target complexes isolated from cell lysate. Following characterization, capture efficiency of the most selective aptamers was planned to be determined by incubating various concentrations of cultured LO-EPCs doped into buffy
coat cell solutions with aptamers immobilized on magnetic beads. This experiment was to be repeated with anti-coagulated whole human blood to assess capture of uncultured (circulating) LO-EPCs. The number and type of captured cells was planned to be assessed both by flow cytometry and traditional culture methods.

Unfortunately, the initial aim to generate aptamers specific toward LO-EPCs was highly challenging and unsuccessful. The selection initially showed promise with enriched affinity toward the LO-EPCs after three rounds, but after five total rounds this selectivity was lost and the nrRNA pool bound substantially better to the buffy coat cells used for negative selection. A reboot of the selection was not attempted, but initial success in early rounds was promising and intimates that future selection attempts employing more conservative approaches are worthwhile and could be fruitful. Subsequent work to characterize aptamers and their targets relied on discovery of LO-EPC-specific aptamers and thus remains proposed. The numerous factors that likely contributed to the failure of the selection and more robust strategies to improve the probability of success in future endeavors are outlined in the discussion.

3.2 Materials and methods

3.2.1 Overview of nuclease-resistant RNA (nrRNA) subtractive cell-SELEX

An overview of the subtractive whole-cell SELEX procedure used to generate aptamers against LO-EPCs is depicted in Figure 13. Briefly, a chemically synthesized,
random, single-stranded DNA (ssDNA) pool was made into double stranded (dsDNA) and then transcribed to nuclease-resistant RNA (nrRNA) possessing 2’OMe-substituted pyrimidines. The random nrRNA pool was gel purified and incubated with LO-EPCs for positive selection during the initial round and all subsequent odd-numbered rounds. Negative selection using freshly isolated whole buffy coat directly followed by positive
selection was performed in even-numbered rounds. After the positive selection step (in all rounds), recovered nrRNA was converted back to ssDNA, amplified by PCR and transcribed into a new nrRNA pool with enhanced binding capacity for LO-EPCs but not buffy coat. This cycle is traditionally iterated with increasing positive selection pressure until no further enrichment in mean binding capacity of the pool to LO-EPCs is observed. Instead of this strategy, HTS was planned to be conducted once substantial enrichment was observed in an attempt to discover aptamers during earlier stages of selection. Details of the methods within this global strategy are outlined below.

3.2.2 Isolation of LO-EPCs and buffy coat cells

LO-EPCs and a double-mutant RNA polymerase capable of transcribing 2’OMe pyrimidine-based nrRNA are not commercially available and thus needed to be isolated and produced in-house. Buffy coat cells also needed to be isolated from freshly collected blood before use to maximize their viability during selections and flow cytometry. As these materials were critically important for the success of this work, details regarding their acquisition are given below.

For isolation of both buffy coat cells and LO-EPCs, 50 mL blood from healthy volunteers or CAD patients (supplied by Dr. Povsic) was collected into vials containing a chelating anticoagulant in strict accordance with guidelines of the Duke Institutional Review Board (IRB). Anti-coagulated blood was then pooled, diluted with buffer,
layered onto a polysaccharide solution and fractionated into plasma, buffy coat and erythrocyte components by density centrifugation. Buffy coat cells were recovered by pipetting and washed several times into media (for LO-EPC isolations) or binding buffer (for negative selection or assessing enrichment). Applications calling for whole buffy coat then utilized the washed cells as called for in those protocols.

LO-EPC isolations continued by seeding the entire buffy coat into a collagen I-coated 6-well plate. The media was very gently changed every day during the initial week using a pipettor, and in following weeks media was changed every other day using a vacuum aspiration. Two weeks after seeding plates, cultures were checked every few days for colonies possessing a classic endothelial cobblestone morphology. Observed colonies were tracked and then passaged normally into T25 flasks upon roughly covering the area afforded by a 4x field of view. Separate colonies arising from a single donor were pooled, expanded and stored typically at passage three.

Several cryopreserved lines of LO-EPCs from previous isolations were initially used, but additional isolations were planned to be performed as necessary throughout the project.

3.2.3 Double-mutant T7 RNA polymerase (YFHA) production and activity assessment

A plasmid containing the gene for YFHA, a T7 RNA polymerase (RNAP) with a polyhistidine tag for purification and two mutations (Y639F, H784A) that permit
efficient incorporation of 2’OMe pyrimidines, was generously gifted from the Sousa and Sullenger labs 247. Bacteria were transformed with this plasmid, expanded in a large culture, induced to express YFHA and then lysed to liberate the enzyme. YFHA was isolated from the lysate using a nickel column that specifically bound the histidine-tagged protein, dialyzed into storage buffer and concentrated in spin filters. Purity of the final product was evaluated on an analytical SDS-PAGE gel and the activity was assessed relative to a commercially available, non-mutant T7 RNAP by monitoring the cumulative amount of nrRNA generated at various timepoints up to a standard reaction length of 8 hours.

3.2.4 Preparation of initial random nrRNA library

A chemically synthesized pool of unique single-stranded DNA (ssDNA) oligomers comprised of a central 40 base randomized sequence flanked by two primer hybridization sites (5’ - TCG GGC GAG TCG TCT G – 40 N – CCG CAT CGT CCT CCC TA - 3’) was used to initiate the selection. A fill-in reaction to generate dsDNA necessary for subsequent transcription was carried out by annealing to the pool a 5’ primer containing a T7 RNAP promoter and then synthesizing the complementary strand using Klenow fragment (large fragment of DNA polymerase I) (Figure 14A). This dsDNA pool was then transcribed to nrRNA containing 2’OMe-derivitized pyrimidines using the YFHA enzyme produced in-house (Figure 14B). Generated nrRNA was
Fill-in Reaction using Klenow Fragment

<table>
<thead>
<tr>
<th>Steps</th>
<th>Synthesis of initial dsDNA pool</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Annealing of 5' primer to random ssDNA template</td>
<td>Sense strand (same seq. as RNA)</td>
</tr>
<tr>
<td>2) Fill in 5' overhangs with Klenow fragment</td>
<td>Antisense (coding) strand</td>
</tr>
<tr>
<td>3) Random dsDNA pool generated</td>
<td></td>
</tr>
</tbody>
</table>

nrRNA Transcription using YFHA Enzyme

<table>
<thead>
<tr>
<th>Steps</th>
<th>Synthesis of nrRNA pool</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Double Mutant RNAP (YFHA)</td>
<td></td>
<td>98 bp DNA</td>
</tr>
<tr>
<td>binds promoter and unzips dsDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) YFHA makes complementary nrRNA to make nrRNA:DNA heteroduplexes and multiple copies of free nrRNA (ssRNA)</td>
<td></td>
<td>98 bp DNA</td>
</tr>
<tr>
<td>3) DNase I digests heteroduplexes and free ssDNA</td>
<td></td>
<td>71 bp RNA</td>
</tr>
<tr>
<td>4) Only nrRNA remains</td>
<td></td>
<td>Mixed</td>
</tr>
</tbody>
</table>

![Diagram](image)

Figure 14: Generation of the starting nrRNA pool containing \( \sim 10^{15} \) unique sequences. (A) \( \sim 10^{15} \) unique ssDNA oligomers (dark green) were annealed to primers (purple) containing a T7 RNA polymerase (RNAP) promoter (orange) and resultant 5' overhangs were “filled in” by Klenow fragment to generate a random dsDNA pool. (B) The dsDNA pool was transcribed to a random nrRNA pool (red) using the double mutant T7 RNAP (YFHA) and a dNTP mix containing 2’OMe-substituted pyrimidines. After the transcription reaction, free DNA and residual heteroduplexes were digested using DNase I to yield only nrRNA.

purified by denaturing polyacrylamide gel electrophoresis (PAGE) to yield the initial pool used for round one of selection (Figure 15).

3.2.5 Performing rounds of positive selection

Positive selection against LO-EPCs was performed during all rounds as depicted in Figure 16. For round one, six nmol initially generated nrRNA pool in binding buffer was denatured at 70°C for 5 min and then allowed to cool to room temperature to create folded nrRNA. The prepared pool was then directly incubated with \( \sim 6 \times 10^6 \) adhered LO-EPCs at \( \sim 85\% \) confluency in petri dishes by rocking for 1 h at 4°C. Unbound
Figure 15: PAGE purification of transcribed nrRNA pools. Generated nrRNA pools were first cleaned by organic extraction with 1:1 phenol/chloroform, concentrated and then ethanol precipitated. The pool was then purified on a large-format 15% denaturing polyacrylamide gel and the band of interest (71 bp) were visualized by UV shadowing, excised, crushed and extracted into TE buffer. Extracted nrRNA was separated from gel fragments and again concentrated to generate the pure nrRNA pool for the next selection round.

sequences were removed by washing 3x with ice-cold wash buffer. Aptamer-cell complexes were mechanically detached via scraping, transferred to a tube and then organically extracted using phenol/chloroform to elute bound sequences. Eluted sequences were buffer-exchanged into TE (tris-EDTA buffer, pH 7.5), concentrated via spin filtration and then ethanol precipitated. The entirety of this recovered and cleaned nrRNA was then resuspended in TE and converted into ssDNA by annealing a 3’ primer and performing a reverse transcription with avian myeloblastosis virus reverse transcriptase (AMV-RT). These sequences were then converted to dsDNA and amplified using PCR, cleaned via another organic extraction and concentrated/buffer-exchanged by spin filtration. A new nrRNA pool for the next round of selection was then generated via transcription with 2’OMe pyrimidines and the YFHA enzyme.
Figure 16: Positive selection against cultured LO-EPCs (all rounds). LO-EPCs previously isolated from CAD patients were grown to ~85% confluence in TCPS petri dishes, washed 3x with wash buffer, and a solution containing a pool of nrRNA (~10^{15} in round one, ~10^{14} in subsequent rounds) in binding buffer was gently rocked over the cells. After this binding reaction, unbound and weakly bound oligos were removed via washing several times. The conditions of the binding reaction and following washes were manipulated to retain only the strongest binders as the selection progressed. Cells were then detached by scraping and organically extracted in phenol/chloroform to elute binders (and endogenous nucleic acid). Isolated nucleic acid was then cleaned up and prepared for reverse transcription. This was performed in all rounds and directly followed negative selection in even-numbered rounds.

Finally, the new nrRNA pool was cleaned up for use in the next round of selection via another cycle of organic extraction, spin filtration, denaturing PAGE purification and ethanol precipitation.
Positive selection pressure was be changed throughout the selection by adjusting parameters of (1) the binding reaction of the pool with LO-EPCs and (2) the subsequent wash step to remove unbound and weakly bound sequences as detailed below.

3.2.6 Performing rounds of positive selection followed by negative selection

Negative selection against freshly isolated buffy coat cells began in round 2 and is summarized by Figure 17. Buffy coat cells were used as this is the population of cells within which circulating LO-EPCs reside, making it imperative for aptamers not to bind strongly to this population. 200 pmol nrRNA pool generated from the last round of selection was folded as for positive selection and then combined with ~10⁷ buffy coat cells in binding buffer for 1 h at 4°C while being agitated on an orbital shaker to prevent cell settling. Cell-aptamer complexes were then pelleted by centrifugation to recover the desirable unbound sequences in the supernatant. This supernatant was then brought to the appropriate volume using binding buffer and directly transferred onto adherent LO-EPCs in petri dishes for positive selection as described above.

Note that while in early rounds (1-5) buffy coat binders were not immediately used, these subtracted sequences were to be eluted, reverse transcribed to cDNA, amplified via PCR and stored (in the same fashion that nrRNA is eluted and processed from LO-EPCs) until HTS was to be performed.
Figure 17: Negative selection against freshly isolated buffy coat cells (even rounds). Freshly collected whole blood is fractionated by density gradient centrifugation and buffy coat cells are isolated by pipetting. This population was washed into binding buffer and 10 M cells were combined with 200 pmol nrRNA pool from the last selection round. This binding reaction was carried out with agitation to maintain the cell suspension at 4°C for an hour. Cells were then pelleted by centrifugation to partition and isolate non-binders of interest in the supernatant, which are then combined with an appropriate volume of binding buffer and directly transferred onto LO-EPCs for positive selection. In later rounds (6-8) of selection, buffy coat binders were eluted and converted to dsDNA as the round-specific “negatively selected pool” for later use in HTS and bioinformatics.

3.2.7 Increasing selection pressure to retain strongest binders

Positive selection pressure was increased over the course of the selection to retain only the strongest binding aptamers and enhance the mean affinity of the nrRNA pool for LO-EPCs. This was done by gradually introducing the following modifications during and after incubation of target cells with the aptamer pool (round-specific changes detailed in Figure 18):

(1) Binding reaction temperature was be increased

(2) Ratio of number LO-EPCs to amount nrRNA was decreased
Figure 18: Selection conditions chosen to increase selection pressure through round 8. Throughout the selection (1) binding reaction and wash conditions became more stringent to retain only the strongest binders and (2) various LO-EPC lines at a range of “ages” and different buffy coat cell sources were used to subtract out binders to any donor-specific markers. Only LO-EPCs isolated from CAD patients were be used for the selection.

(3) Duration of binding reaction was be shortened

(4) Serum was gradually introduced to binding buffer

(5) Wash stringency was increased via increasing their quantity, volume, duration and temperature

Negative selection pressure was intended to remain constant throughout the selection unless the nrRNA pool demonstrated enriched capacity to bind buffy coat cells in addition to LO-EPCs at later stages of selection. In this case, additional negative selections with a greater ratio of buffy coat cells to nrRNA pool was to be introduced.

3.2.8 Biotinylation and fluorescent labeling of aptamers

Aptamers were biotinylated on their 5’ ends by transcribing dsDNA pools saved after each round to nrRNA with the addition of a 5’-biotinylated nucleotide (5’-biotin-guanosine-monophosphate) spiked into the reaction \(^{248}\). Full-length transcripts were
recovered by cleaning up the raw product, performing denaturing PAGE purification as described above and tested to check for biotinylation via an electrophoretic-mobility shift assay (EMSA).

Biotinylated aptamers were fluorescently labeled by incubating with a streptavidin-phycoerythrin (SA-PE) conjugate (2:1 molar ratio of aptamer to SA; theoretically 2 of 4 biotin binding sites on SA occupied) for 20 min at room temperature.

3.2.9 Assessing enrichment via flow cytometry

Figure 19 illustrates preparation of samples for enrichment monitoring by flow cytometry. Stored dsDNA pools from select rounds were transcribed into biotinylated nrRNA and fluorescently labeled with SA-PE. The progress of the selection was monitored by a binding assay in which fluorescently aptamer (200 nM) was incubated with LO-EPCs or buffy coat cells (2.5 M cells/mL) using the binding reaction conditions of the last-performed selection round. Cells were then washed to remove unbound aptamers and flow cytometry was performed to quantify the per-cell fluorescent intensity, which corresponded to the number of aptamers bound per cell. Enhanced binding affinity for target cells is indicated by an increase in the mean fluorescent intensity of LO-EPCs incubated with aptamers from the latest round relative to the unselected initial random pool. Selectivity was assessed by the relative fluorescent signals of LO-EPCs and buffy coat cells.
Figure 19: Procedure used to stain cells with fluorescently labeled aptamers for enrichment monitoring via flow cytometry. 5’-biotinylated nrRNA pools from select rounds were labeled with a streptavidin-PE conjugate and then incubated with LO-EPCs (shown) or buffy coat cells under the binding reaction conditions of the last performed round. Cells were then washed and stored on ice until flow cytometry.

3.3 Results

3.3.1 Double mutant T7 RNAP (YFHA) production and activity assessment

The yield of recombinant YFHA enzyme was spectroscopically determined to be approximately 19.2 mg, or roughly enough for a few hundred selections. Purity was also determined to be exceptional by SDS-PAGE (Figure 10A). Good activity was observed relative to a commercially available, non-mutant T7 polymerase when transcribing both native and nuclease-resistant RNA (Figure 10B), permitting its use in nrRNA transcriptions moving forward.
Figure 20: Recombinantly produced YFHA enzyme was pure exhibited good activity. (A) SDS-PAGE showed that several dilutions (lanes 2-6) of YFHA stock were exceptionally pure, with only a single band of expected size (100 kDa) even when loading the greatest mass of product (lane 2). (B) Activity was assessed by performing transcriptions with commercially available non-mutant T7 RNAP and in-house produced YFHA. Both enzymes had similar trends, with activity dropping off after a couple of hours and overall lower apparent activity for the native enzyme at the added concentration of 20 U / 100 μL. YFHA activity was essentially the same transcribing both native and nrRNA.

3.3.2 Biotinylation assessment via EMSA

Biotinylation of nrRNA was qualitatively confirmed by a gel shift assay (Figure 21). Non-biotinylated nrRNA migrated as expected even when pre-reacted with streptavidin. In contrast, a significant fraction of biotinylated RNA that was pre-reacted with SA exhibited highly retarded migration. This indicated that a large amount of nrRNA was successfully biotinylated and able to bind to SA, making it acceptable for labeling via a SA-fluorophore conjugate.
Figure 21: Biotinylation of nrRNA was successful. A gel-shift assay using denaturing PAGE revealed that migration of biotinylated but not regular nrRNA was retarded when pre-reacted with streptavidin (SA), indicating that biotinylated nrRNA was complexed with SA and consequently migrated based on the mass of the complex rather than the nrRNA alone.

3.3.3 Enrichment after five selection rounds

LO-EPCs from a CAD patient and buffy coat cells from a healthy volunteer were stained with fluorescently labeled nrRNA pools from rounds 1-5 and subjected to flow cytometry to monitor the progress of the selection (Figures 22 and 23). Modest enrichment in binding capacity (apparent affinity) and specificity toward LO-EPCs by round 3 (R3), but this initially encouraging trend was reversed after R4 and by R5 there was no LO-EPC-specific enrichment above background (i.e. relative to the initial random pool, R0) (Figure 24). Unfortunately, reduced enrichment toward LO-EPCs coincided with substantially increased enrichment toward buffy coat cells (across 3 subpopulations), and by R5 at there were more binders per-cell for the second largest buffy coat subpopulation than for LO-EPCs (Figure 24).
Figure 22: Flow cytometric analysis indicated loss of initially observed LO-EPC-specific enrichment by round 5. Binding capacity of nrRNA pools toward LO-EPCs increased through round 3 (R3; light green trace) but decreased to that of the random starting pool (R0, light blue trace) by R5 (pink trace), indicating a drop in the apparent mean affinity of the pool beginning at R4 (dark green trace). These differences are better observed and compared to buffy coat cells in Figure 24.

3.4 Discussion

Aptamers selective toward LO-EPCs were not able to be generated using the chosen selection strategy, forcing subsequent work that hinged upon success of this initial rate-limiting step to also remain uncompleted. While enrichment through first three rounds showed promise with increasing affinity of the nrRNA pool toward LO-EPCS, this trend reversed in following rounds with substantially better binding to buffy coat cells used for negative selection. This unexpected result was actually the opposite of what the selection sought to achieve and signaled that a re-tooled enrichment approach would be required for success.

The success of SELEX is heavily reliant on consistent presentation of a target and
Figure 23: Flow cytometric analysis of multiple buffy coat revealed undesirable enrichment. The entire buffy coat population was gated into three subpopulations as indicated and round-specific per-cell fluorescence was plotted. Binding capacity of nrRNA pools toward buffy coat cells was not substantially changed through R3, but significantly increased in R4 ("main" and "minor" subpopulations) and R5 (all subpopulations). These differences are better observed and compared to LO-EPCs in Figure 24.

efficient propagation of pool sequences that bind that target. Well-defined simple selections against purified, known targets such a small molecule or recombinant protein enable the use of a steady “sink” for binders. Altering the character of the target, for example using the same protein but with a different glycosylation pattern, effectively
Figure 24: Binding capacity toward LO-EPCs increased through R3, then decreased concomitantly with increased binding capacity toward buffy coat cells in R4 and R5. (A) Raw mean fluorescent intensities (MFI) of each cell population across all rounds (R0-R5) including unstained cells (“none” – no nrRNA added). Per-cell fluorescence increased for LO-EPCs through R3 and then decreased in subsequent rounds. Substantial absolute fluorescence of cells in the “major” buffy coat population was seen, indicating a high initial binding capacity for this subpopulation that was preserved through R4 and significantly increased R5 to greater than that for LO-EPCs. (B) Enrichment relative to the unselected starting pool over five rounds. Moderate enrichment for LO-EPCs was observed by R3, but decreased in later rounds as enrichment for all buffy coat cells was observed. (C and D) Relative per-cell fluorescence (C and D are inverses; both shown for clarity) indicate the relative number of binders per cell and provide a sense of specificity of the overall pools. Greater LO-EPC specificity achieved by R3 decreased in later rounds.
reduces the amount of sink for binding sequences and thus fewer binders will be retained and carried over to the next round. This is a form of increased selection pressure and favors retention of the strongest binders that bind both protein variants. However, increasing the selection pressure too quickly in early rounds can cause important sequences to be lost as the pool is not yet sufficiently enriched in high affinity binders. By the same rationale, highly efficient recovery via partitioning bound from non-bound sequences is critical in early rounds and why selection pressure is intentionally kept low until the pool is sufficiently enriched in higher affinity binders. Even taking care to respect this fragility of the early pool and when carried out with experienced hands, it is estimated that only 50% of these relatively simple selections are successful\textsuperscript{249,250}.

By comparison, whole-cell SELEX applied without a priori knowledge of discriminating surface features is notoriously difficult and noisy. Cell surfaces present a myriad of targets whose nature and number can drift as influenced by age, culture conditions, crowding, etc. – even for well-established and immortalized cell lines and when care is taken to prepare cells consistently for use in selections. The breadth of potential targets is often weened down using similar but different cells – e.g. healthy versus diseased cells – for counterselection in which sequences shared between the two cells types are subtracted from the pool. This enriches the pool in sequences selective for
target cells, but this presents and additional step at which sequences of interest are be
easily lost.

In addition, partitioning bound from non-bound sequences centrifugation –
employed in this study and also the most commonly used method – is readily
undermined by the presence of co-pelleted dead cells that act as nucleic acid sinks. In
positive selection steps, this indiscriminately propagates pool sequences that are likely
not selectively for the target cells and can greatly dilute the pool. It has been reported
that the presence of >2% dead cells can delay or prevent enrichment even after 20 cycles
249. As most rare sequences are usually lost during the early stages of selection and due
to domination of PCR artifacts after 12 rounds, dead cells can effectively kill a selection
by diluting sequences that are then further diluted by PCR noise 167, 249, 251. Elution of
bound sequences using phenol/chloroform extraction is also challenging due co-
extraction of native cellular nucleic acid in addition to bound pool RNA, which can also
contribute to PCR artifacts. Thus even with awareness of these issues and working
conscientiously on “idealized” whole-cell selections, the technical challenges posed by
this SELEX method are difficult to overcome and are especially prone to doomng
selections in early rounds as was seen here.

The selection strategy attempted here for LO-EPCs took this already-difficult
method to another level in perhaps one of the most ambitious undertakings possible. In
the case of LO-EPCs, the identity and abundance of aptamer targets on the cell surface are unknown and change while circulating immature cells differentiate to their mature phenotype after isolation and culture in vitro. To compound this challenge, we attempted to “universalize” aptamers during the selection by subtracting out markers not common to all LO-EPCs via the use of cells (1) from different CAD patient donors and (2) at different passage numbers (Figure 18). This deliberate use of a moving target was meritorious but short-sighted and overzealous, as it placed tremendous selection pressure on the pool even in early rounds when its integrity was most delicate. It is suspected that this was a major contributing factor to the failure of the selection.

Another potentially problematic choice was the use of whole buffy coat cells for negative selection. Circulating LO-EPCs are conventionally isolated from buffy coat and thus may have been present in the cell population used for negative selections. LO-EPCs would have acted as sinks and subtracted desired sequences from the pool, delaying delay enrichment or derailing the selection depending on the magnitude of the effect. This possibility was considered but the use of buffy coat was still rationalized as the rarity of LO-EPCs in buffy coat (0.00001%) meant that only about ten cells in the ten million used for negative selection might be problematic. However, given the rarity of sequences in the initial rounds this may have removed potentially important aptamer candidates.
Dead cells were cautiously avoided but potentially still present during positive selections, which as stated could have had a large impact on the direction of the selection. As positive selection was done on adherent LO-EPCs, thorough pre-selection washes should have removed most non-adherent dead cells that could have acted as a non-specific cell sink. Given that as little as 2% of a target cell population can thwart a selection, greater precautions should have been taken to ensure the total absence of dead cells during these binding steps.

Perhaps most directly relevant for downturn in the selection was hastily increased selection pressure. It is actually impressive that, given all of the potential issues for whole cell-SELEX in general and those specific to the LO-EPC selection strategy executed here, there was some mild enrichment seen through round three. It was a modest shift, but apparent pool affinity did increase successively after each round until round four. In round four, both the positive selection incubation and wash temperature were greatly increased from 4°C to room temperature following a negative selection step. As many aptamers bind much more weakly at warmer temperatures, a significant portion of the LO-EPC binding pool may have been discarded at this step. Additionally, the effectiveness of the negative selection step was unknown. Buffy coat binders may not have all been soaked up by the buffy coat cells, and the partitioning efficiency was likely imperfect. Together, these may have substantially increased and
decreased the fractions of buffy coat and LO-EPC binders in the pool, respectfully. PCR amplification for round five and continued application of the warmer washes in addition to utilizing serum to increase selection stringency could have flipped the selection trend on its head at this juncture.

Given the encouraging first few rounds of data and having identified several pitfalls that likely compromised the attempted selection, a few tweaks to the strategy outlined here are expected to impart a much greater probability of success in future LO-EPC selection endeavors. Firstly, a much more gradual increase in positive selection pressure should be applied via (1) consistently using target cells of the same donor at the same passage number and (2) gradually increasing the binding and wash temperature /stringency. Secondly, the cells used for negative selection should be a well-defined artificial buffy coat substitute of ~70% lymphocytes and ~30% monocytes as this closely mimics the composition of natural buffy coat. It should be noted that immature EPCs do possess some monocytic character, so an even safer route would be to use 100% lymphocytes for initial negative selections and gradually increase in the monocyte content as the pool becomes focused in later rounds. It may also make sense to perform an initial negative selection before positive selection on the naïve pool. This is typically not done due to the risk of losing sequence diversity, but given the preference of the pool for buffy coat that emerged here it may be advisable. Thirdly, a positive selection
implementation that guarantees exclusion of dead cells should be used. Instead of using adherent LO-EPCs, cells should be lifted from their growth flasks with gentle trypsin, immediately neutralized and then used for suspension-based positive selection similar to the method shown here for negative selection. The pool should also be fluorescently labeled. After positive selection, cells should be sorted by FACS alongside parallel samples for live/dead cell discrimination and isolate only live cells for subsequent bound nrRNA elution as usual to ensure only LO-EPC selective sequences are retained and propagated \(^{249}\). Finally, bound nrRNA should be eluted from negative selection cells and converted back to cDNA, amplified and stored along with the round-specific DNA pools stored after each positive selection. This would enable collection of HTS data capable of providing great insight into the identification of aptamer candidates via comparison of negative and positive pool sequences \(^{167}\). Together, these suggested modifications greatly increase the chances of successfully identifying LO-EPC aptamers whose value is more than worth the effort due to their tremendously impactful clinical utility as both a diagnostic marker and therapeutic for rapidly developed, non-invasively sourced endothelialized vascular grafts.

### 3.5 Conclusions

This study aimed but ultimately failed to discover nrRNA aptamers selective toward LO-EPCs for the purposes of (1) discovery of novel, uniquely identifying
biomarkers, (2) in vitro diagnostics for LO-EPC-predictive disease and (3) rapid amassment of LO-EPCs via their direct affinity-based capture from whole blood in an ex vivo shunt device. The proposed work was even more zealous than most innately ambitious a priori whole-cell SELEX efforts, but encouraging results in the initial rounds stacked with insightful lessons learned promise a much greater probability of success in a reattempted selection. Methods to characterize aptamers generated in a future LO-EPC selection and assess their ability to capture cells are provided in Appendix B.
4. Antidote-mediated reversal of extracellular aptamer staining

4.1 Introduction

Selective purification of cells is routinely performed and critical for numerous clinical and basic research applications. For example, enrichment of CD34+ cells for the 45,000+ hematopoietic stem cell transplantations performed annually \cite{191-194} or for enrichment of CD8+ cells for adoptive T-cell therapy rely on this technique for isolating therapeutically viable cells \cite{195-198}. Researchers often isolate primary cells to study a particular function or experimental treatment and depend on their unaltered behavior to ensure findings are relevant to native cell function in vivo. A combination of fluorescent monoclonal antibodies – the “gold standard” for specific cell labeling – and fluorescence activated cell sorting (FACS) is the primary workflow for isolating cells of interest \cite{252-254}.

However, lingering antibody stains can be problematic and hamper the usefulness of isolated cells in downstream applications. Often times antibodies bind antagonistically to receptors, disrupting their native signaling behavior in some fashion. Problems can arise when antibodies used for cell purification target a receptor of interest for study or another receptor whose signaling impacts global cell behavior. Residual antibody stains can thus directly or indirectly undermine basic research experiments via altering the function of isolated cells, potentially compromising data validity.
The in vivo utility of cells coated with antibodies is additionally curbed by their inherent immunogenicity. Almost all antibodies are immunogenic due to possessing a Fc region that is identified as foreign by the innate immune system\textsuperscript{255}. The complement system and natural killer cells will recognize infused antibody-coated therapeutic cells as opsonized threats via Fc receptors and activate to induce lysis of the curative cells, rendering the therapy mute. This issue can then snowball by triggering host-versus-graft disease in which an adaptive immune response is mounted against therapeutic cell-specific antigens. Debris from complement- and NK-mediated destruction of therapeutic cells are phagocytosed and presented to B and T lymphocytes that then further augment the attack on the therapeutic cells independently of the presence of foreign antibody. Activation of many other cell types also occurs, secondarily causing inflammation and exerting other negative effects. For these reasons, the use of antibodies for purifying cellular therapeutics is strongly contraindicated.

These problems are well-recognized as methods have been devised to avoid the residual immunogenic antibodies. One strategy is to use negative depletion, in which antibody-labeled undesired cells are removed from a population to yield an impure population significantly enriched in unlabeled desired cells\textsuperscript{256,257}. This technique can be suitable depending on the required purity of the particular application. Another strategy primarily benefitting clinical applications is to purify target cells labeled with
antibodies engineered to lack the immunogenic Fc region[258]. This method yields a pure population of therapeutic donor cells coated with modified antibodies that are invisible to the host immune system, allowing the cells to exert their curative effects assuming the antibodies don’t compromise function. Unfortunately, neither of these approaches guarantees a pure population of uninhibited cells for basic research or clinical use.

A highly promising and alternative approach is to use aptamers for selective cells staining. Aptamers are single-stranded nucleic acids that share the highly selective character of antibodies but have additional assets including being innately non-immunogenic, easily chemically modified and highly stable with long shelf lives. Perhaps the greatest benefit of aptamers is their ease of functional reversibility with matched antidotes. Matched antidotes designed using Watson-Crick base pairing rules are capable of rapidly and selectively neutralizing cognate aptamers to reverse their effects[177, 179, 259]. This powerful technique has shown great promise and efficacy but to date has only been demonstrated with therapeutic aptamers targeting soluble proteins for coagulation control. In principle, the same paired aptamer-antidote system could be used to remove aptamer-based stains after using them to label and isolate target cells. While the residual aptamer stain would not incite an immune reaction to therapeutic cells in vivo, freeing cells of potentially function-altering ligand benefits all applications by ensuring native cell behavior. This concept is illustrated in Figure 25.
To show proof of principle of this approach, we stained A431 epidermoid carcinoma cells with a fluorescently-labeled EGFR-targeting aptamer E07 (Figure 26) and screened for antidotes capable of removing bound aptamer. E07 is a well-characterized aptamer that nicely recognizes A431 cells due to their exceptionally high EGFR expression levels. E07 is a neutralizing aptamer that when bound to EGFR blocks stimulation by agonist EGF and is thus a function-compromising ligand. In addition to monomeric E07, fluorescent multivalent E07-streptavidin (SA) conjugate mixtures and monoclonal EGFR antibodies were used to label cells. The dyed aptamer conjugates were highly stable at 4°C and capable of selective, robust cell staining that was stable during cell sorting. However, monomeric E07 stain stability was poor at warmer temperatures, enabling facile aptamer removal (“destaining”) after FACS even without antidote when exposed to 37°C for short durations. Addition of the most promising antidote to emerge from screening experiments greatly enhanced the rate and extent of destaining. E07-SA conjugate mixtures were significantly more stable than monomeric E07 but were still highly reversible in the presence of antidote. Antibodies were observed to be the most stable stains, but as expected they were not able to be removed. Stability data was corroborated by experiments that assessed receptor function after destaining treatment.
Figure 25: Overview of using an aptamer to selectively stain and sort target cells followed by antidote treatment to remove the aptamer and restore native cell function. Target cells of an initially heterogeneous population are selectively labeled with a surface receptor-specific aptamer (A) and then isolated via fluorescence-activated cell sorting (FACS) (B). In the case of neutralizing aptamers and antibodies, the lingering stain on purified target cells can inhibit native receptor function (C) and confound downstream research and clinical applications. However, subsequent treatment of aptamer- (but not antibody-) stained cells with matched antidotes (D) allows gentle stain removal, restoring native receptor function (E) and enabling intended use of the target cells. The presented work demonstrates proof-of-principle of this technology using the AF488-labeled neutralizing EGFR-binding aptamer E07 and paired antidotes to stain, sort and “destain” epidermoid carcinoma (A431) cells ahead of probing targeted receptor function.
**Figure 26: Predicted secondary structure of E07.** One of two secondary structures predicted to be highly favorable and dominate when folding at 4°C, temperature at which this work is carried out except for the 37°C treatment to destain cells. This structure is also predominantly predicted by the software at 37°C.

E07, E07-SA conjugate mixture and antibody stains all inhibited EGFR stimulation, but antidote-mediated removal of aptamer-based stains restored native behavior of the receptor whereas antibody-stained cells were irreversibly compromised. This work demonstrated that function-blocking aptamers and aptamer-SA conjugates used to label target cells were later removable and yielded purified target cells with uncompromised behavior.
4.2 Materials and methods

Buffers, reagents and experimental samples were maintained at 4°C either on ice or in refrigerated centrifuges during all procedures unless otherwise stated. Care was taken to minimize ambient light exposure of fluorescent samples. Media supplemented only with 1% bovine serum albumin ("media+") was universally used for staining and antidote solutions, washes and flow cytometry buffer.

4.2.1 Cell culture

A431 epidermoid carcinoma cells sourced from the American Type Culture Collection (ATCC) were obtained through the Duke Cell Culture Facility. A431 cells were maintained at 37°C and 5% CO₂ in growth media (high-glucose Dulbecco’s Modified Eagle’s Medium containing L-glutamine, sodium pyruvate, and sodium bicarbonate and 4.5 g/L glucose; Sigma supplemented with 10% non-heat inactivated FBS (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin) per ATCC recommendations. Cells were passaged at 70-90% confluency using 0.25% trypsin-EDTA (Gibco) and split 1:2 – 1:4 into new tissue culture flasks. Experiments utilized A431 cells between passage numbers 32 and 38.

4.2.2 Chemical synthesis of aptamers

E07 and non-specific control C36 RNA aptamers modified with 2’F pyrimidines were produced in house with protected 5’ thiols and 3’ inverted thymidines by solid
phase synthesis on an Expedite 8909 DNA synthesizer (Applied Biosystems). Aptamer sequences are provided in Table 1. Synthesized aptamers were deprotected, HPLC-purified and 5’ labeled with AlexaFluor488 (AF488) using a thiol-reactive AF488-C5-maleimide dye (Invitrogen). Free dye was removed by washing labeled aptamers with 10 mM Tris-EDTA pH 7.5 (TE) in Amicon Ultra spin filters (EMD Millipore). The concentration of dye-labeled aptamer was quantitated using a NanoDrop spectrophotometer (Thermo Scientific).

4.2.3 Biotinylated aptamer transcription and aptamer-streptavidin conjugate preparation

Aptamers possessing 5’-biotinylated eight-nucleotide “tails” (extensions) were transcribed to enable conjugation to AF488-labeled tetrameric streptavidin (AF488-SA; Thermo Fisher). Tails were designed to ensure Mfold software-predicted aptamer secondary structures were not impacted (Figure 27). Double-stranded DNA templates for tailed, biotinylated E07 (TbE07) and C36 (TbC36) containing a T7 RNA polymerase (RNAP) promotor were generated by annealing purchased primers (Integrated DNA Technologies; IDT) and filling in the single-stranded overhangs using Klenow Fragment (NEB). 5’-biotinylated aptamers were transcribed with Y639F mutant T7 RNAP and a ribonucleotide mix containing 2’F pyrimidines doped with a 10-fold molar excess of 5’-biotin-G-monophosphate (TriLink Biotechnologies). Aptamers were purified on denaturing 12% polyacrylamide gels, extracted overnight in TE, desalted in Amicon spin
Table 1: Sequences of aptamers and primers.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Nucleic Acid Type</th>
<th>Sequence</th>
<th>Production</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>E07</td>
<td>2’F C/U RNA</td>
<td>5’-GGA CGG AUU UAA UCG CCG UAG AAA GCA UGU CAA AGC CGG AAC CGU CCT-3’</td>
<td>Chemical synthesis</td>
<td>5’ AF488 3’ inverted T</td>
</tr>
<tr>
<td>C36</td>
<td>2’F C/U RNA</td>
<td>5’- GGC GUA GUG AUU AUG AAU CGU GUG CU AUA CAC GCC T-3’</td>
<td>Chemical synthesis</td>
<td>5’ AF488 3’ inverted T</td>
</tr>
<tr>
<td>TbE07 FP</td>
<td>DNA</td>
<td>5’-GAT AAT ACG ACT CAC TAT AGG GAT TTA GGA CGG ATT TAA TCG CCG TAG AA-3’</td>
<td>Chemical synthesis</td>
<td>-</td>
</tr>
<tr>
<td>TbE07 RP</td>
<td>DNA</td>
<td>5’-GGA CGG TTC CGG CTT TGA CAT GCT TTC TAC GGC GAT TAA ATC CGT CCT AAA TCC C-3’</td>
<td>Chemical synthesis</td>
<td>-</td>
</tr>
<tr>
<td>TbE07</td>
<td>2’F C/U RNA</td>
<td>5’-GGG AUU UA GGA CGG AUU UAA UCG CCG UAG AAA GCA UGU CAA AGC CGG AAC CGU CC-3’</td>
<td>Transcription</td>
<td>5’ biotin</td>
</tr>
<tr>
<td>TbC36 FP</td>
<td>DNA</td>
<td>5’-GAT AAT ACG ACT CAC TAT A GGA AAA TA GGC GTA GTG ATT ATG AAT CGT-3’</td>
<td>Chemical synthesis</td>
<td>-</td>
</tr>
<tr>
<td>TbC36 RP</td>
<td>DNA</td>
<td>5’- GGC GTG TAT TAG CAC ACG ATT CAT AAT CAC TAC GCC TA TTT TCC-3’</td>
<td>Chemical synthesis</td>
<td>-</td>
</tr>
<tr>
<td>TbC36</td>
<td>2’F C/U RNA</td>
<td>5’-GGA AAA UA GGC GUA GUG AUU AUG AAU CGU GUG CU AUA CAC GCC-3’</td>
<td>Transcription</td>
<td>5’ biotin</td>
</tr>
</tbody>
</table>
Figure 27: The designed 5’ 8 base-long tail of E07 (TbE07) does not impact predicted secondary structure. The structure of E07 predicted at both 4°C and 37°C was maintained after addition of a tail to its 5’ end. This extension was added to facilitate SA conjugation.

filters and quantitated via Nanodrop. Sequences of primers and tailed aptamers are provided in Table 1.

Aptamer-streptavidin conjugates were prepared by reacting TbE07 or TbC36 with AF488-SA in molar ratios of 1:1, 2:1, 3:1 or 4:1 (RNA:SA). Aptamers were folded in PBS* by denaturation at 65°C for 5 min followed by passive cooling to ambient

* All PBS contained Ca²⁺ and Mg²⁺ unless otherwise noted
temperature. Conjugates were formed by combining folded aptamers with AF488-SA in PBS at room temperature for 20 min and were directly used without further purification.

4.2.4 Electrophoretic-mobility shift assay (EMSA)

The valencies of aptamer-SA conjugates were characterized by EMSA as previously described. Conjugate samples were prepared as detailed above by varying the amount of aptamer relative to a constant amount of SA. Aptamer-only samples serving as controls contained the same amount of aptamer as the 1:1 conjugate samples. Samples were combined with 2X formamide sample buffer supplemented with 1% SDS and run on an 8% denaturing polyacrylamide gel containing 0.1% SDS at 120V. Aptamer was visualized by staining gels with SYBR Gold (Thermo Fisher) followed by imaging on a ChemiDoc MP (Bio-Rad). Bands corresponding to conjugates of three different molecular weights (smallest, medium and largest) were manually identified using rectangular volumes in Image Lab software (Bio-Rad), and the amount of each aptamer was taken as the background-adjusted intensity of each specified volume. The amount of each size conjugate was normalized to that in the 1:1 conjugate mix for comparison.

4.2.5 Cell staining and flow cytometry

All staining solutions contained 1 mg/mL salmon sperm DNA in media+ to mitigate non-specific binding, and general staining concentrations are provided but
varied as noted in some assays. Aptamer staining solutions contained 500 nM aptamer folded as described for conjugates. Aptamer-SA staining solutions used 2:1 conjugates prepared as stated for 1 μM aptamer and 500 nM AF488-SA. Antibody staining solutions contained 1:50 antibody dilutions. Both FITC-conjugated ICR10 (Abcam) and PE-conjugated D1D4J (Cell Signaling Technologies) monoclonal EGFR antibodies were used for staining.

Experiments utilized cells at 75-90% confluency that had been plated 48-72 hours in advance. Trypsin used to harvest cells was immediately neutralized with growth media upon detachment. Cells were washed once with media+, counted with a hemocytometer, and then 5x10⁵ cells per sample were partitioned into chilled 1.5 mL microcentrifuge tubes. Staining was performed by resuspending the cells 100 uL staining solution (5 M cells per mL) and incubating for 30 min on ice. Stained cells were washed once with 500 uL media+. These samples were either used in further experiments (destaining, stimulation/Western blot) or directly analyzed on BD FACSCalibur or BD FACSCantoII flow cytometers (BD Biosciences). Flow cytometer lasers were always warmed up for a minimum of 30 min prior to analysis to ensure stability during data collection.
4.2.6 Apparent binding affinity determination

The apparent binding affinities of E07 and 1:1, 2:1 and 3:1 TbE07:SA conjugate mixtures were computed from saturation cell binding curves. Cells were stained with 25, 50, 125, 250 or 500 nM E07 or conjugate mixture (based on [SA]), washed and then analyzed by flow cytometry to obtain mean fluorescence intensities (MFI). Apparent dissociation constants (K_D) and saturation binding intensities (B_max) were determined by performing a non-linear regression in GraphPad Prism software to fit flow data to the single-site saturation binding equation MFI = (B_max[aptamer])/(K_D+[aptamer]).

4.2.7 Fluorescence activated cell sorting

Stained samples were filtered through 40 μm cell strainers, resuspended to 10 M cells per mL and kept on ice until sorting. Samples were separate, pure populations of treated cells. Sorting was performed at 30 psi and a rate of 5x10^3 cells per second on a BD DiVa utilizing a chiller to maintain the sample and collection tubes at 4°C. Collection tubes contained 3 mL media+ and approximately 1 mL sheath fluid (PBS without Ca^{2+} or Mg^{2+}) was added per 0.5 M sorted cells. While sample being sorted and collected was chilled, sheath fluid was room temperature. Sorting-associated losses were assessed by independently analyzing samples immediately prior to and after sorting on a BD FACSCalibur.
4.2.8 Antidote synthesis and screening experiments

Single-stranded antidotes 15 bases in length designed to target various regions of E07 were purchased from IDT. Targeted regions were offset by two bases and together the antidotes probed the entirety of the E07 sequence (Figure 28). Antidote sequences are provided in Table 2.

Two screening assays were conducted to assess relative antidote performance. In a blocking assay, cells were stained as described above but 100 nM E07 either in the presence of 100 uM antidote for 30 min at 4°C or 37°C. A more stringent assay evaluated the ability of antidotes to remove already-bound E07 (i.e. “destain”) from stained cells. For the destaining assay, cells were stained with 100 nM E07 for 30 min at 4°C, washed and then incubated with 100 uM antidote in media+ for 30 min at 4°C. After either screen, cells were washed and analyzed by flow cytometry. The reduction in mean fluorescent intensity of antidote-treated cells relative to an untreated stained control served as a metric for antidote effectiveness.

4.2.9 Destaining cells

Stained cells were resuspended in 100 uL media+ with or without antidote A9 (DNA), a purchased 2’OMe RNA (all bases) version of A9 termed mA9 (Biosynthesis, Inc.), or a scrambled control antidote sA9 (DNA) at concentrations that varied
Figure 28: Antidote screening targeted entire sequence of E07. Fifteen base-long DNA antidotes (A1 – A17) were used to probe nearly the entire length of the E07 sequence in two-based increments. Antidote target regions are highlighted in green, and the predicted loop-forming regions often critical for aptamer binding are bolded. The 3’ “T” denotes an inverted thymidine added for aptamer stability that is present only on chemically-synthesized E07.

depending on the experiment. All samples were simultaneously transferred from ice to a water bath at 37°C for durations up to 30 min. Samples were immediately placed back on ice after the destaining treatment. Cells were washed and then either analyzed by flow cytometry or subsequently used in stimulation assays.

4.2.10 Cell viability assays

Viability of unfixed cells that had been stained, sorted and then destained using a variety of treatments was evaluated by using a LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher) according to the instructions of the manufacturer. Treated
Table 2: Sequences of screened antidotes.

<table>
<thead>
<tr>
<th>Antidote</th>
<th>Sequence</th>
<th>Note: mA9 is composed of 2'OMe bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>5'-TAC GGC GAT TAA ATC-3'</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>5'-TCT ACG GCG ATT AAA-3'</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td>5'-TTT CTA CGG CGA TTA-3'</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>5'-GCT TTC TAC GGC GAT-3'</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td>5'-ATG CTT TCT ACG GCG-3'</td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td>5'-ACA TGG TTT CTA CGG-3'</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td>5'-TGA CAT GCT TTC TAC-3'</td>
<td></td>
</tr>
<tr>
<td>A8</td>
<td>5'-TTT GAC ATG CTT TCT-3'</td>
<td></td>
</tr>
<tr>
<td>A9</td>
<td>5'-GCT TTG ACA TGC TTT-3'</td>
<td></td>
</tr>
<tr>
<td>mA9</td>
<td>5'-GCU UUG ACA UGC UUU-3'</td>
<td></td>
</tr>
<tr>
<td>A10</td>
<td>5'-CGG CTT TGA CAT GCT-3'</td>
<td></td>
</tr>
<tr>
<td>A11</td>
<td>5'-TCC GGC TTT GAC ATG-3'</td>
<td></td>
</tr>
<tr>
<td>A12</td>
<td>5'-GTT CCG GCT TTG ACA-3'</td>
<td></td>
</tr>
<tr>
<td>A13</td>
<td>5'-CGG TTC CGG CTT TGA-3'</td>
<td></td>
</tr>
<tr>
<td>A14</td>
<td>5'-GCG ATT AAA TCC GTC-3'</td>
<td></td>
</tr>
<tr>
<td>A15</td>
<td>5'-CGG CGA TTA AAT CCG-3'</td>
<td></td>
</tr>
<tr>
<td>A16</td>
<td>5'-GAC GGT TCC GGC TTT-3'</td>
<td></td>
</tr>
<tr>
<td>A17</td>
<td>5'-AGG ACG GTT CCG GCT-3'</td>
<td></td>
</tr>
<tr>
<td>sA9</td>
<td>5'-ATC TAT TGT GTT CGC-3'</td>
<td></td>
</tr>
</tbody>
</table>

samples were then analyzed by flow cytometry. Unstained sorted cells that had been heated at 65°C for 20 min served as a positive (dead cell) control for gating.

4.2.11 EGF stimulation assays and Western blotting

Cells were left unstained or stained with PE-labeled D1D4J neutralizing monoclonal antibody against EGFR (Cell Signaling Technologies), E07, or E07-SA (2:1 conjugate mixture). Cells were either sorted and then destained or destained immediately. Destaining was performed for 5 min at 37°C in media or 5 uM mA9,
stimulated with 5 nM EGF in media+ for 15 min on ice and then lysed by resuspension in radioimmunoprecipitation (RIPA) buffer containing phosphatase and protease inhibitor cocktails (Thermo Fisher). Lysate was either used immediately or frozen at -80°C until use.

The amount of protein in crude lysate was quantified using a bicinchoninic acid (BCA) assay kit according to the instructions of the manufacturer. Crude lysate samples containing equal amounts of protein were prepared in Laemmli sample buffer containing 10% β-mercaptoethanol, boiled for 5 min at 95°C and then run on 4-15% denaturing polyacrylamide gels (Bio-Rad) in Tris/glycine/SDS buffer at 300V. Electrophoresed samples were blotted onto low-fluorescence polyvinylidene (PVDF) membranes with the Trans-Blot Turbo Transfer System and Transfer Packs (Bio-Rad) using the default setting for transfer of high molecular weight proteins. Membranes were blocked with 5% (w/v) bovine serum albumin in PBS containing 0.05% Tween 20 (PBST) and incubated overnight at 4°C with primary antibodies for total EGFR and pEGFR (Cell Signaling Technologies). Membranes were washed 3x for 5 min with PBST on a rocker and then treated with appropriate fluorophore-conjugated secondary antibodies for detection of total EGFR and pEGFR in separate fluorescent channels without spectral overlap. Blots were washed in the same fashion and then imaged on a Chemidoc MP equipped with green and red LEDs that enabled fluorescent multiplexing.
(Bio-Rad). Bands corresponding to total EGFR and pEGFR were manually identified using rectangular volumes in Image Lab software (Bio-Rad), and the amount of each protein was taken as the background-adjusted intensity of each specified volume. The pEGFR/total EGFR ratio for each sample was normalized to the average ratio for stimulated unstained cells, which served as the positive control for native, uninhibited stimulation.

4.2.11 Statistics

Data were analyzed by one-way variance (ANOVA) and the Tukey-Kramer post doc test in JMP software to establish significant differences between experimental conditions where appropriate. Significance was assumed at p < 0.05.

4.3 Results

4.3.1 Best performing antidotes targeted predicted loops of E07

When performed at 4°C, staining with E07 in both the presence of antidote (Figure 29A) and before antidote treatment (Figure 29B) only slightly reduced the amount of bound aptamer relative only-stained cells despite excessively high antidote concentrations (100 μM). Even more muted effects were observed conducting these screens at lower antidote concentrations (1 and 10 μM; data not shown). Dramatically improved antidote performance was achieved at 37°C with significantly decreased
Figure 29: Antidote screening revealed loop-targeting antidote A9 as most promising candidate. A431 cells were stained for 30 min with C36 (random control RNA) or E07 in either the absence or presence of 1000-fold molar excess (100 μM) of antidote at either 4°C or 37°C. Samples stained in the absence of antidote were subsequently washed and resuspended in antidote under the same conditions as for staining. Unstained, C36-stained and non-antidote treated E07-stained cells served as controls. Washed samples were then analyzed by flow cytometry and the fraction of the mean fluorescence intensity (MFI) relative to the non-antidote treated E07 samples were plotted. A reduction in MFI indicated antidote-mediated blocking of aptamer binding or removal of bound aptamer. Staining both in the (A) presence and (B) absence of antidote at 4°C only modestly reduced the amount of bound E07, with antidotes targeting predicted loop region being most effective. However, staining in the presence of antidote at 37°C (C) dramatically reduced the amount of bound E07 using antidotes that targeted predicted loop regions. (D) Antidote A9 emerged as the best candidate from these screening assays. Its targeted and was used for subsequent experiments.
bound E07 (Figure 29C). Antidotes targeting predicted loop regions of E07 were consistently the most effective. One of the best performing antidotes across all assays, A9, was selected for use in further experiments (Figure 29D).

**4.3.2 Antidote enhanced removal of bound E07**

E07 was relatively stable on stained cells at the temperature of 4°C maintained during staining and sample handling, but 37°C treatment readily liberated bound E07 as during antidote screening. Destaining cells to 21% of their initial E07-attributed fluorescent intensity over duration of 30 min was achieved with media+ alone (Figure 30). However, the rate and degree of E07 removal was greatly enhanced in the presence of 100 μM A9. This concentration of A9 enabled maximal destaining (6.8% of initial value) by 10 min. These results are representative of several other destaining experiments performed with similarly high antidote levels. The originally selected, full-length E07 aptamer (93 bases) was observed to be lost at the same rate in media+ only, indicating that the instability of E07 was not conferred by truncation (data not shown).

**4.3.3 2’OMe RNA antidote was more potent than DNA**

A 2’OMe RNA version of antidote A9, termed mA9, was more potent than its DNA-based counterpart (Figure 31). The antidote variants were similarly competent destaining agents at ≥ 5 μM, but at lower concentrations mA9 was significantly more capable of removing bound E07.
**Figure 30:** Antidote A9 enhances removal of E07 from cells over media only at 37°C. E07-stained A431 cells resuspended in either media or antidote (100 μM A9) incubated at 37°C removed bound E07. Greater destaining was achieved with longer durations at 37°C both cases, but antidote greatly increased the rate and amount of E07 removed compared to media only.

### 4.3.4 Antidote-enhanced destaining was specific

Enhanced removal of bound E07 was only observed with sequence-specific antidote. The ability of the scrambled A9 sequence variant, sA9, to destain cells was compared to A9 and mA9. sA9 did not augment destaining over media+ only even at the excessively high concentration of 25 μM, suggesting that the action of designed antidotes A9 and mA9 was highly specific (Figure 32A). The specific potency of these antidotes relative to the controls is highlighted by normalizing antidote-facilitated E07 losses to those in media+ only (Figure 32B).
Figure 31: Antidote mA9 (2’OMe RNA) is more effective than A9 (DNA) at lower concentrations. The potency of DNA-based antidote A9 and its 2’OMe-based counterpart mA9 were compared in a titration experiment. E07-stained A431 cells were resuspended in media containing A9 or mA9 in concentrations ranging from 50 nM to 25 μM and incubated at 37°C for 10 min. Destained cells were washed, analyzed by flow cytometry and the relative reduction in fluorescence corresponding to fold greater E07 removal was plotted for each antidote concentration. Flow curves for (A) A9-treated and (B) mA9-treated cells show that while both antidote types offered similar performance at higher concentrations (≥ 5 μM), mA9 removed substantially more bound E07 at lower concentrations (< 5 μM). The higher potency of mA9 is well visualized by examining the fold greater E07 removal as a function of antidote concentration (C).
Figure 32: Antidotes A9 and mA9 specifically enhance E07 removal. Non-specific destaining was assessed by comparing the effectiveness of A9 and mA9 to a random-sequence control antidote (sA9). E07-stained cells were incubated with media only or each antidote type at 37°C for various durations up to 10 minutes, washed and analyzed by flow cytometry. The reduction in fluorescence corresponding to removal of bound E07 over time is shown in (A), and the enhancement of E07 removal over media only is highlighted in (B). Even at the high antidote concentration of 25 μM, the control antidote sA9 did not improve removal of bound E07 over media only.

4.3.5 Formation of higher valency conjugates was favored by increased aptamer to SA ratios

Multivalent aptamer-SA conjugates were formed by reacting E07 and C36 possessing 5' biotinylated tails (TbE07 and TbC36) with AF488-labeled tetrameric SA. EMSAs revealed that higher aptamer:SA molar ratios resulted in a larger proportion of higher molecular weight conjugates (Figure 33). This presumably corresponded to higher valency conjugates via greater saturation of the four SA sites available for binding.
Figure 33: **Higher valency aptamer-streptavidin conjugates were generated using higher molar ratios of aptamer to streptavidin.** The valency of aptamer-streptavidin (SA) conjugates prepared with different molar ratios of biotinylated aptamer to SA was characterized by electrophoretic mobility shift assays (EMSAs). C36 or E07 with 5’ biotinylated tails (TbC36 and TbE07) was reacted with tetrameric SA with four biotin binding sites in 1:1, 2:1, 3:1 or 4:1 molar ratios of aptamer to tetramer. Free RNA (1:0) and conjugate samples containing equal amounts of SA were then separated by electrophoresis on polyacrylamide gels that denatured the RNA but preserved integrity of the SA tetramers. TbE07 (A) and TbC36 (B) RNA was then visualized by staining gels with SYBR Gold followed by imaging. The smallest (blue), middle (green), and largest (orange) MW conjugates were respectfully assumed to be monovalent, bivalent and trivalent TbE07-SA. Intensities of bands corresponding to each conjugate size were quantified with software and then normalized to the combined intensity of all conjugates for that sample (C and D). TbE07-SA samples prepared from 1:1 and 2:1 TbE07:SA were virtually the same, with more monovalent than bivalent conjugate and very little trivalent conjugate. Higher ratio TbE07-SA samples of 3:1 and 4:1 were also similar, with each having less monovalent conjugate and more bivalent and trivalent conjugates than the lower ratio preparations. TbC36-SA samples exhibited a similar trend, although the yield of higher valency conjugates better at lower ratios and less dramatic at higher ratios.
biotinylated aptamer. Smallest, medium and largest conjugates were respectively thought to be monovalent, bivalent and trivalent. E07-SA conjugate mixtures prepared from 1:1 and 2:1 TbE07:SA were virtually identical, consisting of ~60% monovalent and ~35% bivalent conjugate (Figure 33C). Ratios of 3:1 and 4:1 TbE07:SA yielded a greater proportion of higher valency multimers, with both reaction mixtures composed of ~32% monovalent, ~49% bivalent and ~18% trivalent conjugates. C36-SA conjugates paralleled this pattern, but a better yield of higher valency conjugates was obtained at lower ratios (Figure 33D). Note that all conjugate mixtures also contained unincorporated monomeric tailed aptamer. Subsequent destaining and EGF stimulation experiments utilized unpurified 2:1 aptamer:SA conjugate mixtures for comparison with E07 monomer.

4.3.6 Higher ratio E07:SA conjugate mixtures exhibited higher apparent affinities

Apparent affinities of E07-SA conjugate mixtures increased with higher ratios of TbE07:SA, but increased affinity was accompanied by a concomitant decrease in apparent saturation (Figure 34). For comparison, the apparent affinity of E07 (directly labeled monomer; 62.7 nM) was closer to that of the 1:1 conjugate mixture (85.1 nM) than the 2:1 mixture (14.7 nM).
**Figure 34:** Higher ratio E07:SA conjugate mixtures possessed higher apparent affinities but lower apparent saturation. A431 cells were stained with various concentrations of E07 only or E07-SA conjugate mixtures prepared by reacting 1:1, 2:1 or 3:1 biotinylated E07 with tetrameric streptavidin. Stained cells were washed, analyzed by flow cytometry and their fluorescence corresponding to the amount of bound E07 or E07-SA was plotted to determine apparent binding affinities (dissociation constants; KD) and cell surface saturation (total number of receptors; Bmax). Conjugate mixtures prepared from higher E07:SA ratios had higher apparent affinities, but lower apparent saturation. Unconjugated E07 had a higher affinity than the 1:1 conjugate mixture but lower affinity than the 2:1 and 3:1 mixtures.

### 4.3.7 E07-SA conjugate mixtures were more stable than E07 during sorting and destaining

The use E07 and E07-SA conjugate mixtures as reversible stains for cell sorting was validated and compared to different EGFR-binding monoclonal antibodies (ICR10 and D1D4J). All stain types yielded stable fluorescent cell populations distinct from unstained cells using a chiller to maintain samples being sorted at 4°C. Fluorescence of samples being sorted was compared at the beginning and end of each sort, and no drift was detected for any stain type (data not shown).
However, losses did occur during sorting despite actively chilling initially cold sample collection tubes (Figure 35). Losses attributed to sorting were determined using flow cytometry to analyze samples immediately before and after FACS. E07 losses (46%) were nearly double those of the E07-SA conjugate mixture (25%), while both antibodies were more than twice as stable as the conjugate at ~11% losses each.

Sorted cells were then destained in media+ or antidote mA9 to further assess stability and reversibility (Figure 36). Antibody stains remained highly stable and seemingly irreversible as cells labeled with ICR10 or D1D4J exhibited minimal losses during destaining in media+ (Figure 36A). E07-SA conjugate mixture was markedly more stable during destaining in both media+ and 500 nM mA9 than E07 monomer (Figure 36B and C). A higher mA9 concentration of 5 μM bridged the gap in removal of E07 and E07-SA conjugate mixture, with only a small fraction of each remaining after destaining for 30 min (Figure 36C). Overall, these data mirror and corroborate the stability data derived from sorting-attributed stain losses.

4.3.8 High cell viability was preserved during sorting and destaining

The viability of sorted, destained A431 cells was very high (> 95%) and not impacted by any treatment variable including stain type, the use of antidote or destaining duration (Figure 37).
Figure 35: Significantly more monomeric E07 than E07-SA conjugate mixture was lost during sorting. Stained A431 cells were analyzed by flow cytometry immediately before and after FACS to examine losses that occurred while sorting. E07 was the least stable with losses of 46%, followed by E07-SA (2:1 E07:SA conjugate mix) with 25% losses and EGFR antibodies with about 11% losses each. Each group is significantly different from all others (p < 0.0001 for *, ** and ***).

4.3.9 Destaining rescued native stimulability of aptamer-stained cells

Destaining treatment successfully reversed aptamer-induced inhibition of EGFR stimulation, while antibody-induced inhibition was irreversible. EGF binding to EGFR results in phosphorylation of its intracellular domain to generate pEGFR, and this stimulation event nucleates a series of downstream signals that modulate cellular function. The degree of stimulation caused by EGF was assessed by determining the relative phosphorylation levels (pEGFR/total EGFR) of cells in different treatment
Figure 36: Destaining sorted cells highlighted additional stability of E07-SA conjugate over E07 monomer. A431 cells stained with E07, E07-SA (2:1 E07:SA ratio conjugate mixture) or one of two different monoclonal EGFR antibodies were sorted by FACS and then destained in media, 500 nM mA9 or 5 μM mA9 at 37°C for up to 30 min to remove bound E07 or E07-SA. Cells were then analyzed by flow cytometry and reduction in fluorescence of each stain type corresponding to its removal was plotted. EGFR antibodies ICR10 and D1D4J were the most stable stains, experiencing only slight losses over 30 min at 37°C in media (A). E07-SA was substantially more stable than E07 both in media only and in antidote, but higher concentration of antidote (5 μM vs. 500 nM) effectively removed the bound conjugate mixture over 30 min (B and C).

Quantitative Western blotting probing for total EGFR and pEGFR was used to evaluate protein levels.

Stimulation of unsorted cells is shown in Figure 38. EGF stimulation of cells stained with E07 (Figure 38A), E07-SA conjugate mix or neutralizing antibody D1D4J (Figure 38B) and destained in media+ for 5 min was significantly reduced compared to an unstained control. Destaining instead with 5 μM mA9 sufficiently removed EGFR-blocking aptamer to restore stimulation to the level of unstained cells. Notably, a two-hour incubation on ice in after destaining in media+ only also rescued stimulability of cells stained with E07 but not E07-SA conjugate mixture, additionally implicating
Figure 37: High cell viability was maintained after sorting and destaining for all treatments. Live/dead assays indicated that high A431 cell viability was maintained regardless of staining and destaining treatment. Prior to sorting cells were either left unstained or stained with EGFR antibody (D1D4J), C36, E07, SA-C36 (2:1 C36:SA conjugate mixture) or E07-SA (2:1 E07:SA conjugate mixture). Sorted cells were then destained for 0 or 30 min in media only or 5 μM mA9. Unstained, sorted cells that were untreated or heat-treated (65°C for 15 min) respectively served as (A) live and (B) dead controls. No treatment significantly impacted viability relative to the live control, with all conditions sustaining viabilities >95% (C and D).

Reduced stability of the monomer relative to the conjugate mixture (data not shown). In contrast, antibody binding and thus compromised EGFR function was irreversible.

Recoverable stimulability was also demonstrated for sorted cells by conducting the same assay (Figure 39). Negative controls C36 and C36-SA conjugate mix were included to
Figure 38: Only destaining with antidote restored ability of unsorted aptamer-stained cells to be stimulated with EGF. Quantitative Western blotting enabled quantification of EGF-induced stimulation of A431 cells via probing for phosphorylated (pEGFR; red) and total EGFR (green). * denotes stimulation with EGF; media DS = destained with media only; mA9 DS = destained with 5 μM mA9. Antibody and unstained cells were destained with media only. The degree of stimulation was taken as the ratio of the band intensities of pEGFR and total EGFR, as EGF binding triggers autophosphorylation of EGFR to generate pEGFR. Stained or unstained cells were destained for 5 min at 37°C in media or 5 μM mA9, stimulated with 5 nM EGF for 15 min on ice and then lysed in the presence of phosphatase and protease inhibitors. Crude lysate samples containing equal amounts of protein were prepared, separated by denaturing polyacrylamide gel electrophoresis and blotted onto a membrane. The membrane was blocked with albumin and incubated overnight at 4°C with primary antibodies for total EGFR and pEGFR. Secondary antibodies were used for detection and the blots were imaged. Band intensities corresponding to total EGFR pEGFR were quantified by software and the pEGFR/total EGFR ratio for each sample was normalized to the average ratio for stimulated unstained cells, which served as the positive control for native, uninhibited stimulation. Destaining (A) E07 and (B) E07-SA (2:1 E07:SA conjugate mix) with media yielded significantly reduced stimulation compared with unstained cells. However, destaining with mA9 removed enough (A) E07 and (B) E07-SA to enable native stimulation levels in line with those of unstained cells. The neutralizing EGFR antibody D1D4J irreversibly suppressed stimulation to a greater degree than any other treatment (B). Each group is significantly different from all others (p < 0.0001 for *, **, *** and ****).
Figure 39: Antidote treatment was necessary after sorting for restored EGF stimulability of E07-SA-stained but not E07-stained cells. Quantitative Western blots were also performed on A431 cells that had been sorted via FACS. Destaining, stimulation, lysing and Western blotting procedures to probe for phosphorylated EGFR (pEGFR; red) and total EGFR (green) were identical to those used for unsorted samples. * denotes stimulation with EGF; media DS = destained with media only; mA9 DS = destained with 5 μM mA9. Antibody and unstained cells were destained with media only. Destained C36 and SA-C36 (2:1 C36:SA conjugate mix) samples (A) did not inhibit stimulation of cells relative to the unstained positive control, but C36 and SA-C36 samples did stimulate cells to slightly different degrees (p = 0.012 for ** vs. ***). Unlike unsorted samples, destaining sorted E07 samples (B) with media was sufficient to restore stimulation to native levels. In contrast, sorted E07-SA samples (2:1 E07:SA conjugate mix) (C) still required antidote to rescue cell stimulability.

show that the presence of non-specific aptamer stain and streptavidin did not alter stimulation levels from unstained cells (Figure 39A). After sorting, sufficient E07 was removed after destaining with media+ only to bring stimulation in line with unstained cells and mA9-destained cells (Figure 39B). However, samples stained with the
relatively stable E07-SA conjugate mix still required destaining with mA9 to rescue stimulation (Figure 39C).

4.4 Discussion

This proof-of-principle study is the first demonstration of using aptamers and matched antidotes to reversibly stain cells. Aptamer stains provided stability required for purifying labeled cells via FACS at 4°C, but subsequent destaining via a short incubation at 37°C gently removed the bound aptamer. Antidotes specifically enhanced the rate of destaining, and 2′OMe RNA antidote was more potent than its DNA counterpart. Unpurified aptamer-streptavidin conjugate mixtures exhibited higher valency, avidity and stability than monomeric aptamer, but higher antidote concentrations still readily destained conjugates from cells. High cell viability was maintained across all experimental conditions despite cells being subjected to a variety of stresses and kept cold for long durations. Importantly, stimulation of the receptor targeted by the aptamer was restored upon antidote-mediated destaining, rescuing native behavior of cells sorted with a function-compromising ligand. Antibodies targeting the same receptor also provided good stability for sorting, but their permanence irreversibly crippled the ability of isolated cells to be stimulated.

It was found early in the study that E07 staining was only stable at low temperatures (Figure 30). While this property was actually an asset for its removal, it
was critical to keep E07-stained samples cold until completion of sorting to preserve a strong fluorescent signal that clearly identified the cell population of interest.

Spontaneous loss at 37°C of the minimized E07 aptamer used in this study was also observed for its full-length parent sequence (data not shown), implying the instability was not imparted through truncation. Additionally, other studies have reported high off-rates of aptamers that reduce their utility for several applications including cell purification and targeted delivery for therapeutics\textsuperscript{263}.

Maintence of E07-stained samples at 4°C was also important for inhibiting endocytosis of bound aptamer, a process that readily occurs at physiological temperature. The complex of E07 and its target EGFR is readily internalized upon binding\textsuperscript{264,265}, which would prevent its removal during destaining. In addition to improving the stability of bound E07, low temperatures also hinder endocytosis due to substantially reduced fluidity of the cell membrane. Sustaining samples at 4°C thus kept E07 stable on the extracellular surface to (1) provide robust signal during sorting and (2) maximize downstream aptamer removal.

In line with this rationale, antidote screening experiments to neutralize or remove bound E07 were initially performed at 4°C. The low cost of commercially produced DNA made well suited (versus modified RNA) for initial screening experiments, especially given the extremely high concentrations (100 μM) ultimately
used. Even at that excessive concentration, 15-mer antidotes that together sampled the entirety of the E07 sequence failed to substantially block binding or destain already-bound aptamer at 4°C (Figure 29 A and B). Lower concentrations had an even more muted effect (data not shown). As avoiding aptamer internalization was not paramount for the purposes of antidote screening, blocking was then performed at 37°C with dramatically better results (Figure 29C). These data and those acquired during cold screenings together indicated that antidotes targeting the predicted loop structures of E07 were most effective, with antidote A9 having been one of the most effective and chosen as the lead antidote moving forward. This finding of loop-targeting antidotes being highly effective was consistent with successful antidotes developed in previous work.

It was then realized that the antidotes may not have been functional in the cold assays due to being structured. Fifteen-to-twenty base antidotes had successfully been used in the past to rapidly neutralize aptamers at physiological temperature, but at 4°C these antidotes are predicted to be structured. In fact, a cursory survey of potential antidotes against E07 revealed that antidotes greater than 10 bases long on average had structure at that temperature (data not shown).

This finding prompted the attempt of an alternative strategy to destain cells at 4°C. Instead using a single larger antidote that would be folded when cold,
combinations of smaller antidotes were tested that together targeted larger regions but whose individual small sizes avoided structuring when cold. Groups of two or three antidotes seven or eight bases long were screened, as DNA this length was predicted to be capable of annealing to its target while not forming secondary structure (data not shown). In theory, this system should have been able to destain cells at 4°C by avoiding the pitfalls that plagued larger antidotes. Only partial success was observed in practice, as short antidote combinations were capable of blocking E07 binding when cold, but they only performed about as well as the best performing longer antidotes at the real test of removing already bound aptamer from cells (data not shown).

In light of this and the established effectiveness of antidote A9 at physiological temperature, short-term 37°C destaining treatment was employed in attempt to remove bound aptamer prior to endocytosis. While heat alone did remove E07 from cells (79% after 30 min), the presence of antidote (100 μM) greatly accelerated removal (97% after 10 min) (Figure 30). This enhancement was found to be specific, as a still-excessively high concentration of control antidote possessing a randomly scrambled A9 sequence did not impact the rate or extent of destaining relative to media+ only (Figure 32). Endocytosis was also assessed and found to be very minor at approximately 4% after 10 min in media+ and 3% in sA9, which is significantly less than the 23% after 30 min reported by another group. The relatively rapid rate of E07 removal in the presence
of A9 and mA9 competed favorably with the rate of internalization, further reducing endocytosis to about 1.5% (data not shown).

Lower concentrations of antidote A9 and its 2’OMe RNA analog mA9 were also tested in order to seek conditions that conserve reagent but still maximize the destaining rate. The minimum concentration of A9 that preserved its best observed destaining power was 5 μM, and mA9 was similarly effective at this concentration. However, its higher potency enabled mA9 to preserve this maximal effectiveness at concentrations up to 10 fold lower (Figure 31). This greater potency has routinely been harnessed in development of antidotes for regulating aptamer therapeutics in vivo and is due to the higher binding affinity of 2’OMe to complementary bases (0.5-0.7°C higher Tm per nucleotide) compared to DNA. Using modified RNA antidotes with non-canonical bases (e.g. 2’F, 2’OMe) is a necessity motivated by the significantly higher nuclease stability in plasma that these bases confer. For in vitro applications like the one demonstrated here where nucleases are not a concern, DNA antidotes offer a financially compelling alternative even if significantly more is needed to achieve the desired effect.

Aptamer instability was also combated by utilizing multimeric E07-streptavidin conjugate mixtures in addition to keeping samples cold. Conjugates were generated by reacting biotinylated E07 or control C36 with AF488-labeled tetrameric streptavidin that
theoretically supports decoration with four aptamers. To avoid steric effects that might impact aptamer binding upon conjugation, “tailed” aptamers (TbE07 and TbC36) with biotinylated 5’ extensions were used (Figure 27). Additional cushion was provided by a six carbon spacer between the biotin and the first nucleotide of the tail.

Reactions of biotinylated aptamer with SA yielded mixtures of unreacted aptamer and conjugates possessing a range of valencies, with an TbE07:SA molar ratio of 3:1 appearing as a threshold for the production of substantially more multivalent conjugate (Figure 33). This result is consistent with other studies working with aptamer-SA constructs that found the 3:1 to be optimal. It is theorized that, despite biotin-SA being the strongest non-covalent bond known and an excess of available aptamer binding sites, maximum valencies of only two to three are possible due to (1) steric crowding and (2) electrostatic repulsion on SA as aptamer is added. This theory explains the large amount of unincorporated aptamer in even the 1:1 mixture, the spectrum of conjugate species afforded and the requirement of excess aptamer to drive the yield of multivalent constructs.

The compositions of the unpurified conjugate mixtures explain the apparent binding affinity and saturation observed when used to stain cells. Since the amount of TbE07 was increased relative to a constant amount of SA to prepare conjugate mixtures, more unreacted TbE07 was present in higher ratio mixtures despite better incorporation
observed by EMSA. The greater fraction of non-fluorescent unreacted TbE07 at higher ratios better competed for binding and consequently reduced the amount of bound fluorescent conjugates, lowering the apparent saturation (Figure 34). Unlabeled competitor should not have impacted apparent binding affinity, so the greater apparent affinities of higher ratio conjugate mixtures accurately reflected their increased content of higher avidity constructs. While a greater difference in affinity of 3:1 versus 2:1 mixtures was expected given its higher average valency, there are diminishing returns on avidity for greater than bivalent conjugates\(^{183,184}\). As unpurified conjugate mixture stains were compared to E07 in subsequent experiments, a ratio of 2:1 was selected for its similar apparent affinity but reduced competition with unlabeled, lower affinity competitor TbE07 that would greatly impact EGF stimulation assays.

Despite its composition, significantly improved stability approaching that of monoclonal EGFR antibodies was achieved using unpurified conjugate reaction mixture of E07-SA relative to monomeric E07 (Figure 36). Destaining experiments reflected this as a substantial reduction in aptamer lost over time at 37°C, and functional assays showed much stable blocking of EGFR that prevented stimulation by agonist EGF (Figure 38). This improved average stability granted by the conjugate mixture was even more apparent after cell sorting (Figure 39). The large amount of relatively unstable unreacted TbE07 monomer and monovalent aptamer-SA conjugate that bound in
addition to the more stable multivalent constructs was lost at the same rate as E07-stained samples, freeing a large fraction of EGFR for stimulation by EGF and reducing the average inhibition conferred by conjugate mixture treatment. Considering this, the observed results are quite impressive.

Yet, the greater average stability in media+ bestowed by the conjugate mixtures was entirely defeatable at higher antidote concentrations (5 μM) (Figure 36). Interestingly, the observation that reduced antidote concentrations were less effective at destaining conjugates implies they are crosslinking EGFRs on the cell surface. TbE07 conjugated to SA but not associated with EGFR at the time of antidote treatment should be neutralized and consequently the local concentration of aptamer available for rebinding should be reduced. Thus, only multivalent conjugates simultaneously bound to more than one EGFR receptor prior to antidote treatment should provide additional stability over the monomer. Such multivalency-enabled crosslinking is routinely observed for antibodies, and the dimerization of EGFR is a well characterized.

It was also promising that extremely high cell viability was maintained after sorting and destaining regardless of the treatment type (Figure 37). The procedure of detaching, staining, sorting, and destaining involves many stresses including multiple centrifugation steps and samples being kept cold for several hours. While A431 cells may be relatively robust and the viability data are only for necrosis and not apoptosis,
the results are still very favorable and speak to the gentle nature of reversible aptamer-based cell staining.

### 4.5 Conclusions

The ability to reversibly stain cells without compromising their viability or function is a valuable, versatile tool with important implications for both the lab and clinic. The highly encouraging data presented here serve as proof of principle of this technology. Even more promising is the prospect of using purified multivalent aptamer constructs, which are predicted to perform at least as well as monoclonal antibodies while retaining the aptamer-exclusive benefit of antidote-mediated reversibility. This methodology serves as an adaptable platform for other aptamer-antidote pairs that are able to suit a myriad of research and clinical applications.
5. Conclusions and perspectives

5.1 Dissertation summary

The goal of these projects was to develop a set of innovative diagnostic and therapeutic tools for vascular grafts and cell purification. First, a novel method of assessing vascular graft thrombogenicity in vitro was established and provided groundwork for discerning the failure mechanism of lumen-associated thrombi. Next, the generation of aptamers for selective LO-EPC capture and biomarker discovery was ambitiously undertaken with initially promising results and a strong plan to complete the work under a revised strategy. Finally, an established aptamer and a matched antidote were used to reversibly stain cells for purification without compromising their downstream utility as traditionally used antibodies can.

The introduced method of assessing vascular graft thrombogenicity in vitro is the first to use pressure buildup upstream of a clot as a metric to quantify the physical interaction between the grant lumen and a maturing thrombus. The pressure within a closed tubing system was continuously monitored as clotting solutions cured to varying maturities were purged from small-diameter ePTFE grafts or Tygon graft mimics. Maximum pressures achieved prior to purging the coagulum from the graft served as the primary experimental endpoint and an indicator of resistance to flow, with maximum purging pressures increasing with clot maturity. Well-defined fibrin glue
solutions provided consistent purging pressures as a function of curing duration in both non-permeable Tygon and permeable ePTFE. Platelet-rich plasma and whole blood also provided consistent results in Tygon, but their relatively variable and long clotting times led to less consistent results when using permeable ePTFE. This approach provided insight into how blood flow resistance is influenced by a number of clinically relevant factors, such as the level of vessel occlusion and the physical nature of the resident coagulum.

The chosen subtractive whole-cell SELEX strategy to generate aptamers selective toward LO-EPCs ultimately failed but was initially promising. Each round yielded improved affinity of the pool toward LO-EPCs relative to buffy coat until round four when stronger selection pressure was applied. This reversed the enrichment trend and by round five the pool substantially favored binding to buffy coat over LO-EPCs, effectively ending the selection attempt. However, the encouraging results observed during the first three rounds suggest that there is hope for generation of LO-EPC aptamers with an amended approach that avoids the pitfalls which likely contributed to this unfavorable ending. The experience gained and lessons learned here contributed to formulation of a more conservative proposed strategy that was outlined in the discussion of Chapter 3.
Aptamer-antidote pairs were successfully used as reversible, selective labeling agents for cell purification, demonstrating proof-of-concept for this powerful and elegant strategy. The stability of bound E07, a neutralizing aptamer against EGFR, was found to be relatively low at temperatures above 4°C. This turned out to be an asset, as it and enabled E07 stains to be stable for cell sorting when cold but also facilitated destaining of cells at 37°C following purification. The best-performing DNA antidote to emerge from screening assays was capable of significantly enhancing the rate and extent of aptamer removal relative to media only. A 2’OMe RNA version of this antidote was more potent and was more effective at lower concentrations. To create more stable yet still reversible aptamer stains, multivalent constructs were generated by reacting biotinylated E07 with fluorescently labeled streptavidin theoretically capable of supporting four constituents. Unpurified reaction mixtures were directly used in experiments and contained a variety of predominantly monovalent and bivalent conjugate species in addition to unincorporated, non-fluorescent E07. Despite the impurities and low average valency of the conjugate mixtures, they were significantly more stable than monomeric aptamer at both warmer temperatures and lower antidote concentrations. Higher antidote concentrations were substantially more effective at removing the conjugate mixture from cells. Critically, both forms of the aptamer stain blocked stimulation of their target EGFR, but destaining treatment with antidote
recovered stimulability to that of non-stained cells. In contrast, antibodies were unable to be removed and permanently compromised cell function. This work validated aptamer-antidote technology as a robust yet reversible alternative to conventional antibody-based stains for selective cell purification.

5.2 Implications of research

The immense, unmet and growing need for readily available high-performance small-diameter vascular grafts has fueled intense research and development of novel prosthetics that will require extensive validation prior to being clinically available. In vitro assays such as the one presented here represent cost-effective, rapid initial screening methods to that offer great utility for identification of the most promising synthetic graft technologies ahead of ex vivo and in vivo testing. As most in vitro assays focus on characterization of particular aspects of biocompatibility, a number of different assays should be employed to gain the most insight into potential graft performance. For example, an assay that examines the mechanical properties of the graft and associated thrombus could be combined with one revealing detailed molecular hemocompatibility data. Together their results could predict the graft technologies worth of pursuing ahead of animal testing.

The results of the selection for aptamers against LO-EPCs spoke to the delicate and challenging nature of directed evolution work and to that of subtractive whole-cell
SELEX in particular. While the project remains incomplete, its results signal what adjustments should be made to favor success in future selection attempts. Utilizing LO-EPCs at various passage numbers from different donors at each positive selection step presented a deliberately inconsistent target, which was an overzealous attempt to “universalize” aptamers by throwing out sequences that did not bind to every type of LO-EPC without having first achieved sufficient enrichment. This imposed a strong selection pressure that likely diluted the pool in LO-EPC binders on top of the other potential pitfalls discussed in Chapter 3. Impressively, enrichment was seen until round three despite this and this provides strong encouragement that discovering aptamers selective for LO-EPCs is possible with some tweaking. It is only when even stronger positive selection pressure was deliberately applied in rounds four and five that pool affinity for LO-EPCs dropped. These results indicate that a more conservative selection strategy can succeed and should be attempted due to the impact aptamers against LO-EPCs would possess. The aptamers able to be generated with a more conservative approach promise (1) discovery of novel, uniquely identifying biomarkers that place these cells in a clearer context, (2) sensitive in vitro diagnostics that accurately enumerate LO-EPCs to monitor cardiovascular disease and (3) rapid amassment of LO-EPCs for angiogenic cellular therapies and expeditious fabrication of endothelialized synthetic vascular grafts.
Aptamers and matched antidotes for non-destructive, reversible cell staining offer an unrivaled cell purification platform. This technique is impactful because the uncompromised utility of cells purified using it. The staining-sorting-destaining workflow was successful in removing nearly all of the aptamer and had no negative impact on cell viability or function. Multivalent aptamers, even in the form of a heterogenous mixture containing a large fraction of monomer, offered near-antibody stability but were also completely reversible via antidote treatment. Imperfect destaining does not contraindicate aptamer‑sorted cells for therapeutic use in vivo as they are not immunogenic like antibodies. Since aptamers can be raised against nearly any target using SELEX and match antidotes are effortless designed, this technology is extendable to virtually any cell type.

This method could also be useful for serially sorting a cell population for multiple biomarkers using a single fluorophore. Research environments with limited resources may only have a single-laser (color) sorter but need to purify a specific subpopulation of cells defined by a set of surface markers. Using antibodies, cells could only be sorted for one marker as the stain is permanent and sorting the population again with another antibody possessing the same fluorophore would be messy or impossible. However, sets of aptamer-antidote pairs all labeled with the same fluorophore would
provide a clean slate after each sort to allow sequential sorts in the same channel and eventually allow the population of interest to be isolated.

Reversible cell staining technology is also useful for enhancement of targeted in vivo imaging. Administering labeled aptamer intravenously enables its concentration in targeted tissues for imaging. A follow-up treatment with antidote treatment to neutralize the aptamer more readily removes non-specifically bound aptamer contributing to background signal and improves contrast to better visualize the targeted tissue of interest. The Sullenger lab has accomplished this with a thrombin-binding aptamer and matched antidote to image clots in vivo with good initial success, suggesting that the same system extended to tissue via cell-binding aptamer-antidote pairs would also work well for this application.

The same concept of using antidote for more specific in vivo signal could be applied toward drug delivery as well. Having an antidote on board systemically to curb non-specific chemotherapeutic effects, higher concentrations of drug could be delivered locally to tumors for potentially enhanced therapeutic effect. As antidotes are able to modulate aptamer function in a dose-dependent manner, titration with drug and/or antidote presents a powerful treatment modality.
5.3 Future work

The pressure-based in vitro thrombogenicity assay presented here could be modified to provide more insight into the clot failure mechanism. While the presented version of the system could provide a quantitative measure of resistance to flow, it was only able to do so as a lumped parameter. At the maximum purging pressure, clots failed due either to (1) shearing off of the lumen, resulting in complete or partial ejection of the thrombus, or (2) medial clot rupture in which the center of the clot was blown out but the thrombus remained adhered to the lumen wall. Data gathered with the system in its current form could not be analyzed to delineate these failure modes. Qualitatively, visual inspection of exposed graft lumens revealed that longer curing times generally resulted in a medial clot rupture for all clotting solutions (fibrin glue, PRP and whole blood), suggesting lumenal adhesion strength was greater than bulk clot strength.

A robust, quantitative and precise method to assess this endpoint would provide valuable supplementary data that may be predictive of graft behavior in vivo. For example, such information may correlate with the tendency of a graft to nucleate thrombi and shed them as emboli or to develop a strongly adherent thrombus. One potential non-visual quantitative technique may be to apply oscillatory shear stress to assess wall adhesion strength \(^{271}\). It is possible that future modification of the presented system to an opposing syringe pump configuration could enable this type of testing,
providing an additional metric by which to quantify the physical interaction between a developing clot and the graft lumen.

This oscillatory approach could also be tailored to create dynamic flow conditions as opposed to the static conditions used in the current iteration of the system. Static conditions present a more stringent test of thrombogenicity as platelet aggregation is promoted, but it is more physiologically relevant and to utilize flowing blood. Opposing syringe pumps and blood reservoirs on either side of the graft would permit continuous laminar flow over a graft to combat platelet settling and localized thrombus deposition. An interesting approach that could tease out meaningful data for additional insight would be to automatically modulate the oscillation rate based on real-time pressure data as clots develop. That is, laminar flow could be maintained until flow resistance increases to a threshold predictive of the presence of a thrombus. At various clot maturities beyond that (as in the current pressure system), the oscillatory behavior could be adjusted to assess luminal adhesion of the clot \(^{271}\). These modifications would bring additional utility to this in vitro vascular graft assessment method.

Based on the initially encouraging results of the LO-EPC selection and the enormous utility offered by aptamers selective toward these cells, this work should be rebooted and follow a more conservative selection strategy that would greatly improve the chances of success. Having surmised the nature of the failed selection attempted
here, proposed methods to successfully complete the selection and to characterize successfully generated LO-EPC aptamers are described in Chapter 3.4 and Appendix B, respectively. The suggested methods can at the very least provide some perspective and serve as a starting point for planning a new approach. It is highly recommended that any aptamer work be performed in an environment of experts that can regularly provide guidance in both technique and strategy for the best chances of success.

The success of the reversible aptamer stains can be further solidified by pursuing an array of avenues. Prior to publication of the work presented here, experiments to destain sorted cells and demonstrate restored function will be repeated using purified (not mixtures) bivalent and trivalent E07-SA conjugates. These assays will provide a much cleaner picture of conjugate performance relative to antibodies. Based on the results to date, purified conjugates are expected to offer antibody-level stability while maintaining the ability to be reversed with antidotes. Additional aptamer-antidote pairs and cell lines should be explored to make an even stronger case for the technique. Basic research would benefit from the serial sorting described earlier, which could also be performed to demonstrate plausibility. Finally, tunable in vivo imaging and targeted chemotherapy for tumors using E07 and mA9 would highlight the power of this technique for clinical applications other than cellular therapeutics.
Appendix A: License for previously published work

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 24, 2017

This Agreement between Michael D Nichols ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number 4055430055836
License date
Licensed Content Publisher John Wiley and Sons
Licensed Content Publication Journal of Biomedical Materials Research
Licensed Content Title Coagulation-induced resistance to fluid flow in small-diameter vascular grafts and graft mimics measured by purging pressure
Licensed Content Author Michael D. Nichols, Rewa Choudhary, Santhisri Kodali, William M. Reichert
Licensed Content Date Oct 22, 2013
Licensed Content Pages 10
Type of use Dissertation/Thesis
Requestor type Author of this Wiley article
Format Print and electronic
Portion
Full article
Will you be translating?
No
Title of your thesis / dissertation
Diagnostics and Aptamer-based Therapeutics for Synthetic Vascular Grafts and Reversible Cell Stains
Expected completion date
Mar 2017
Expected size (number of pages)
150
Requestor Location
Michael D Nichols
702 Edenberry Drive
DURHAM, NC 27713
United States
Attn: Michael D Nichols
Publisher Tax ID
EU826007151
Billing Type
Invoice
Billing Address
Michael D Nichols
702 Edenberry Drive
DURHAM, NC 27713
United States
Attn: Michael D Nichols
Total
0.00 USD
Terms and Conditions
TERMS AND CONDITIONS
This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a
particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, and any CONTENT (PDF or image file) purchased as part of your order, is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is
granted subject to an appropriate acknowledgement given to the author, title of the
material/book/journal and the publisher. You shall also duplicate the copyright notice
that appears in the Wiley publication in your use of the Wiley Material. Permission is
also granted on the understanding that nowhere in the text is a previously published
source acknowledged for all or part of this Wiley Material. Any third party content is
expressly excluded from this permission.

With respect to the Wiley Materials, all rights are reserved. Except as expressly
granted by the terms of the license, no part of the Wiley Materials may be copied,
modified, adapted (except for minor reformatting required by the new Publication),
translated, reproduced, transferred or distributed, in any form or by any means, and no
derivative works may be made based on the Wiley Materials without the prior
permission of the respective copyright owner. For STM Signatory Publishers clearing
permission under the terms of the **STM Permissions Guidelines** only, the terms of the
license are extended to include subsequent editions and for editions in other languages,
provided such editions are for the work as a whole in situ and does not involve the
separate exploitation of the permitted figures or extracts, You may not alter, remove or
suppress in any manner any copyright, trademark or other notices displayed by the
Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security,
transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted
to you hereunder to any other person.

The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS
LICENSORS AND WAIVED BY YOU.

WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.

You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.

IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED
REMEDY PROVIDED HEREIN.

Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.

The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party’s right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.

This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY’s prior written consent.

Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.

These terms and conditions together with CCC’s Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and
WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties’ successors, legal representatives, and authorized assigns.

In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state’s conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby
consents and submits to the personal jurisdiction of such court, waives any objection to
venue in such court and consents to service of process by registered or certified mail,
return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in
Subscription journals offering Online Open. Although most of the fully Open Access
journals publish open access articles under the terms of the Creative Commons
Attribution (CC BY) License only, the subscription journals and a few of the Open Access
Journals offer a choice of Creative Commons Licenses. The license type is clearly
identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy,
distribute and transmit an article, adapt the article and make commercial use of the
article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the
original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License
The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.
Appendix B: Proposed methods for evaluating LO-EPC aptamers

Characterization of LO-EPC aptamers

Overall goal and approach

The goal of these proposed methods is to identify sequences in the pool that have become enriched through SELEX and characterize their binding properties to discover the aptamer candidates with the highest affinity and specificity toward LO-EPCs. Framed around a recent cell-SELEX method utilizing HTS and bioinformatics software, these tools can be used together to identify sequences that are relatively abundant in nrRNA pools eluted from LO-EPCs relative to those eluted from buffy coat cells from rounds 6-8. These potentially important sequences should then be chemically synthesized and their apparent binding affinities to LO-EPCs and buffy coat cells should be determined and compared to identify aptamer candidates most specific to LO-EPCs from various donors. Binding targets of the most selective aptamers should then be elucidated via mass spectrometry, potentially revealing a uniquely identifying LO-EPC biomarker.

Proposed methods

High-throughput sequencing (HTS)

Both positive and negative selection aptamer pools from all rounds should be prepared for sequencing on an Illumina HiSeq 2000 using a TruSeq DNA Sample Prep
kit as described by Meyer et al.\textsuperscript{167} Briefly, aptamers should be ligated to adapters needed for flow cell immobilization within the sequencer and then PCR amplified. Prepared samples should be submitted to a sequencing core and run on the an instrument such as the Illumina. If using the Illumina, the Single Read 200 Base Pair protocol should be utilized to obtain 15+ million reads from each pool.

**Identification of aptamer candidates using bioinformatics**

In-house MATLAB software can be written to implement an algorithm conceived by Lei Chen and depicted by Figure 40. This algorithm could be used to filter the raw data set and enumerate the number of times a unique sequence appears in each pool sample. An “enrichment ratio” could then be calculated as the ratio of abundance of a given sequence within pools eluted from LO-EPC (positive selection) to those eluted from the cells of the well-defined buffy coat mimic (negative selection). Sequences with the top 20 enrichment ratios from each round would be potentially important aptamer candidates and should be chemically synthesized for use in binding assays.

**Assessment of aptamer binding affinities**

Apparent dissociation constants (K\textsubscript{D,app}) of toward both LO-EPCs from multiple donors and buffy coat cells should be determined as described elsewhere.\textsuperscript{153, 166} Briefly, 2x10\textsuperscript{5} cells should be incubated with various fluorescently labeled aptamer candidates at concentrations ranging from 0-500 nM under binding conditions used for
HTS sequencing read of positive and negative selection pools from rounds 6-8

- Closely matched header and footer?
  - No → “Bad” reads
  - Yes → Exactly 71 bases between header and footer?
    - No → “Candidate” reads
    - Yes → “Good” reads

Count and rank frequency of unique sequences

Figure 40: Algorithm to filter potentially important aptamer sequences from HTS data. Erroneously sequenced oligos from selection pools should be removed from data to leave correct sequences of full-length reads. This filtered pool should then be processed to enumerate and rank the frequency of unique sequences in pools from positive selection relative to pools from negative selection (subtracted sequences) for a given round. Unique sequences most enriched in the positive pool relative to the negative should be classified as potentially important aptamer candidates.

Positive selection of round 8. Cells should then be washed and the mean fluorescent intensities of stained aptamers should be quantified by flow cytometry. A negative control sample of fluorescently labeled random pool oligos incubated with cells should also be run to determine background fluorescence of each cell type. Background
fluorescence should be subtracted from that of aptamer candidates, and mean fluorescence intensity resulting from specific binding should be plotted as a function of aptamer concentration. Apparent dissociation constants (K_D) and saturation binding intensities (B_max) can determined by performing a non-linear regression fit flow data to the single-site saturation binding equation MFI = (B_max[aptamer])/(K_D+[aptamer]).

**Elucidation of aptamer targets (biomarker identification)**

Biomarkers to which LO-EPC aptamers bind can be elucidated using mass spectrometry as detailed by Berezovski et al. Briefly, LO-EPC aptamers should be biotinylated and immobilized on magnetic beads. These beads should be incubated with non-enzymatically detached LO-EPCs in selection buffer, washed and then incubated in lysing buffer. Beads should then be washed and bound proteins can be removed by incubation in urea for 30 min at 0°C. Detached protein can be recovered by retaining the beads with a magnet and then digested with sequencing-grade trypsin. This peptide mixture should be extracted, purified using C18 column-packed pipette tips and then eluted with an acid/solvent. Finally, mass spectrometry should be performed and MASCOT software used to scour proteomic databases and determine target protein identities.
Assessment of the ability of the aptamers to capture cultured and uncultured (circulating) LO-EPCs

Overall goal and approach

The goal of methods described here is to probe the capacity of biotinylated LO-EPC aptamers to capture both cultured (previously isolated and expand) and uncultured (circulating) LO-EPCs from Buffy coat and whole blood using streptavidin-coated magnetic beads. Magnetic beads are a common and efficient method to isolate cells of interest from heterogeneous cell mixtures, representing a pragmatic application of LO-EPC aptamers. This platform should be used to evaluate the capture efficiency of the top 5 most specific nrRNA aptamers as depicted in Figure 41.

Cultured LO-EPCs spiked into Buffy coat cell solutions should serve as a well-defined mimic of whole blood to emulate competition for binding sites on magnetic beads and initially test aptamer capturing ability. This experiment should then be repeated using heparinized whole blood doped with cultured LO-EPCs. Finally, the ability to capture uncultured LO-EPCs present in freshly isolated Buffy coat and collected whole blood should be explored.

Proposed methods

Evaluating aptamer capture efficiency of cultured LO-EPCs

Aptamers with the top 5 determined LO-EPC selectivities should be biotinylated and be immobilized on streptavidin-coated magnetic beads. Well-defined solutions of
Figure 41: The magnetic bead-based method to assess capture efficiency of LO-EPCs aptamers. SA-coated magnetic beads should be pre-reacted with 5'-biotinylated aptamers, washed to remove residual nrRNA and combined with mixtures of LO-EPCs doped into well-defined buffy coat solutions (depicted) or whole blood. Captured cells should be isolated from non-bound cells by application of a magnet, liberated via trypsinization and cultured prior to characterization.

cultured LO-EPCs should be prepared by spiking these cells at concentrations of 0, 5, 25, 100 and 500 cells/mL into freshly isolated buffy coat cells at 2 M cells/mL in binding buffer. Aptamer-conjugated beads should be incubated with each of these cell solutions under the binding reaction conditions used for last-performed round of positive selection. Bead-cell complexes should then be isolated with a magnet and cells should be detached using trypsin. Detached cells should be cultured for 72 h to recover surface proteins damaged by trypsinization and permit proliferation. Captured cells then
should be non-enzymatically detached and stained for monocytic markers (CD14 / CD11c / CD45) and the best consensus for LO-EPCs (CD31 / CD34 / CD146) prior to characterization and enumeration by flow cytometry.

This experiment should then be repeated with freshly collected, heparin-anticoagulated whole CAD blood doped with cultured LO-EPCs at the same concentrations noted above.

If the capture efficiency of cultured LO-EPCs is inadequate to isolate these cells when doped at the lowest concentration of 5/mL, a greater amount of aptamer-coated magnetic beads relative to the cell population should be used in attempt to scavenge these rare cells. This cultured LO-EPC experiment should be performed first to assess the viability of moving forward with the more demanding capture of circulating LO-EPCs.

**Assessing utility of aptamers to capture uncultured LO-EPCs**

The experimental set-up for capturing circulating LO-EPCs should be identical to that used capture cultured LO-EPCs without these cells doped into buffy coat or whole blood. After the binding reaction, magnetic bead-cell complexes should be transferred to collagen I-coated 24-well plates similar to those used for conventional LO-EPC isolations and any bound cells will be released by trypsinization. These cultures should be maintained normally for 2 weeks and checked daily for signs of normal LO-EPC
morphological development and the emergence of LO-EPC colonies. Colonies that emerge should be expanded to P3 per the protocol for conventional LO-EPC isolations before being stained with the same markers listed in above and characterized by flow cytometry.

If magnetic beads prove to be too inefficient to capture cultured LO-EPCs at the scarcity found in circulation, this assessment should be reframed around using an aptamer-coated microfluidic device to efficiently capture these cells. Dr. Steven Soper, a professor of chemistry at UNC Chapel Hill and an expert in affinity-based cell capture diagnostics, developed the aptamer immobilization technique that has been successfully used to detect rare circulating tumor cells\textsuperscript{190}. As this microfluidics-based detection is intended for diagnostics, this alternative path to assessing aptamer utility would still be in the context of a clinically relevant application.
References

1. American Heart Association (American Heart Association, Dallas, TX; 2009).


29. Wong, G. et al., Vol. 47 608-615 (


121. Yoder, M.C. & Ingram, D.A. The definition of EPCs and other bone marrow cells contributing to neoangiogenesis and tumor growth: is there common ground for understanding the roles of numerous marrow-derived cells in the neoangiogenic process? *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer* **1796**, 50-54 (2009).


130. Kim, S.-W. et al. Human peripheral blood-derived CD31+ cells have robust angiogenic and vasculogenic properties and are effective for treating ischemic vascular disease. Journal of the American College of Cardiology 56, 593-607 (2010).


197


226. Povsic, T.J. (Division of Cardiology, Duke University Medical Center, Durham, NC 27710. 2011.


Biography

Michael Douglas Nichols was born on August 7, 1984 in Atlanta, Georgia. He earned a Bachelor of Science degree in Biomedical Engineering at Washington University in Saint Louis from 2003 to 2007, and he stayed to earn a Master of Science in the same discipline while working in the biomaterials laboratory of Donald L. Elbert. His Masters thesis work focused on (1) the development of thin hydrogel coatings to improve the biocompatibility of blood-contacting materials and (2) manipulating the phase-separation behavior of polyethylene glycol polymers to generate modular scaffolds for tissue engineering. After defending his MS thesis in 2009, Michael began pursuit of a doctoral degree in Biomedical Engineering at Duke University under the guidance of William “Monty” Reichert. While in the Reichert lab, Michael received an American Heart Association Pre-doctoral Research Fellowship and was awarded an R21 grant from the National Institutes of Health to support his work.

Publications


