Development of Novel Therapeutic and Diagnostic Approaches for Atherosclerosis

A dissertation presented to
the faculty of
the Russ College of Engineering and Technology of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

Sudhir P. Deosarkar
March 2010

© 2010 Sudhir P. Deosarkar. All Rights Reserved.
This dissertation titled
Development of Novel Therapeutic and Diagnostic Approaches for
Atherosclerosis

by
SUDHIR P. DEOSARKAR

has been approved for
the Department of Chemical and Biomolecular Engineering
and the Russ College of Engineering and Technology by

______________________________
Douglas J. Goetz
Professor, Chemical and Biomolecular Engineering

______________________________
Dennis Irwin
Dean, Russ College of Engineering and Technology
ABSTRACT

DEOSARKAR, SUDHIR P., Ph.D., March 2010, Chemical Engineering

Development of Novel Therapeutic and Diagnostic Approaches for Atherosclerosis

(138 pp.)

Director of Dissertation: Douglas J. Goetz

Atherosclerosis is an inflammatory disease that affects major arteries of the vasculature. Extensive research on atherosclerosis has indicated that among other inflammatory markers, vascular cell adhesion molecule-1 (VCAM-1) is a major player in atherosclerosis development and progression. VCAM-1 has been shown to be elevated at vascular sites prone to atherosclerosis. The elevated expression of VCAM-1 takes part in the adhesion and transmigration of leukocytes across the endothelium. This leads to the formation of atherosclerotic plaques which are the most common cause of adverse cardiovascular events. Hence, this doctoral work was motivated by the need to improve current diagnostics and therapeutics for atherosclerosis wherein the elevated expression of VCAM-1 was exploited for the development of novel therapeutic and diagnostic approaches for atherosclerosis.

In the first study, a VCAM-1 ligand (α-VCAM-1, an antibody to VCAM-1) was conjugated to polymeric particles and their adhesion to the vasculature was probed using a mouse model of atherosclerosis. Specifically, their adhesion to the aorta and the femoral artery was studied. Results from this study demonstrated that the ligand conjugated polymeric particles exhibit an avid and focal adhesion to sites of
atherosclerosis. It was also observed that the particle adhesion is a function of the location within a plaque, position along the length of the aorta and the type of the vascular bed. Particles conjugated with α-VCAM-1 exhibited significantly higher adhesion (as high as 5-fold higher) to the aorta of atherosclerotic mice compared to their adhesion to the aorta of control mice. Additionally, a 2.5-fold higher adhesion was observed for α-VCAM-1 particles to the plaque regions of the aorta compared to the non-plaque regions of the aorta. These results demonstrate that the ligand conjugated polymeric particles can selectively target sites of atherosclerosis and may potentially serve as the vehicles for delivering drugs or imaging agents to atherosclerotic sites.

In a separate study, a real-time PCR based assay was developed to detect and characterize the endothelial cells spiked into whole blood. For this purpose, human umbilical vein endothelial cells (HUVEC) were used as model endothelial cells. This project is motivated by the clinical setting in which blood sample from atherosclerotic patients could potentially contain endothelial cells as a result of vascular damage. Hence, in this study, the use of two endothelial markers viz., MCAM (a marker for endothelial cells) and VCAM-1 (a marker for “the activated state” of the endothelial cells) was explored. Two parameters were defined for assessing the inflamed state of the endothelial cells viz., endothelial VCAM-1 to MCAM ratio (EVMR) and endothelial cell activation index (ECAI). EVMR is a measure of the extent of VCAM-1 expression relative to MCAM expression in endothelial cells whereas ECAI is a measure of the extent of activation of endothelial cells. ECAI values as high as 2000 were obtained in the case of blood spiked with TNF-α treated HUVEC. This indicates that there is nearly a 2000-fold
higher amount of VCAM-1 mRNA in the blood spiked with TNF-α treated HUVEC compared to the blood spiked with untreated HUVEC. Thus, the dual marker approach developed in this study was able to effectively detect activated endothelial cells in whole blood.

Finally, the use of a mathematical model to analyze particle adhesion to atherosclerotic plaques was explored. The results of this analysis indicated that, in addition to the common parameters such as the Peclet number and the Damkohler number, the stenosis height has a substantial effect on the adhesion of particles within the plaque region.

In summary, this dissertation is a step forward towards the development of novel therapeutic and diagnostic approaches for atherosclerosis.

Approved: _____________________________________________________________

Douglas J. Goetz

Professor, Chemical and Biomolecular Engineering
ACKNOWLEDGMENTS

This dissertation would not have been possible without the help, support and guidance from my mentors, teachers, colleagues, friends and family. I owe my deepest gratitude to my advisor, Dr. Douglas J. Goetz, for his exceptional guidance, help and encouragement in my professional endeavors. I wish to extend many thanks to Drs. Ramiro Malgor and Harshad Sakalkar for teaching me critical surgical skills for in vivo experiments. I would like to express my sincere gratitude to Dr. Klaus Ley at the University of Virginia for allowing me to visit his laboratory to learn new in vivo techniques. I wish to thank our collaborators Drs. Justin Hanes and Jie Fu at The Johns Hopkins University for their timely supply of reagents necessary for this work. I am grateful to Drs. David Tees and Darin Ridgway for their help and guidance for the mathematical aspect of this work. I am thankful to Christopher Lewis and Vijayanand Nadella for their help with the real-time PCR technology. It has been a pleasure working with all my colleagues at the Konneker Research Center. I wish to sincerely thank Drs. Leonard Kohn, Kelly McCall, Frank Schwartz and all the members of the Kohn and McCall lab for the lively discussions during our group meetings. I am thankful to Drs. Monica Burdick, Darin Ridgway, Ramiro Malgor and David Tees for agreeing to serve on my dissertation committee. A very special note of appreciation and gratitude goes to my parents Mr. Purushottam Deosarkar and Mrs. Shakuntala Deosarkar for their exceptional support and encouragement.
To my parents
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>3</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>6</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>7</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>11</td>
</tr>
<tr>
<td>CHAPTER 1: ATHEROSCLEROSIS AND MOLECULAR MARKERS FOR THE DEVELOPMENT OF NOVEL THERAPEUTICS AND DIAGNOSTICS</td>
<td>13</td>
</tr>
<tr>
<td>Atherosclerosis and its role in severe cardiovascular events</td>
<td>13</td>
</tr>
<tr>
<td>Role of VCAM-1 in atherosclerosis</td>
<td>15</td>
</tr>
<tr>
<td>Site specific targeting in atherosclerosis</td>
<td>17</td>
</tr>
<tr>
<td>Blood based diagnostics for atherosclerosis</td>
<td>20</td>
</tr>
<tr>
<td>CHAPTER 2: POLYMERIC PARTICLES CONJUGATED WITH A LIGAND TO VCAM-1 EXHIBIT SELECTIVE, AVID AND FOCAL ADHESION TO SITES OF ATHEROSCLEROSIS</td>
<td>24</td>
</tr>
<tr>
<td>Introduction</td>
<td>24</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td>31</td>
</tr>
<tr>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td>CHAPTER 3: DETECTION OF INFLAMED ENDOTHELIAL CELLS IN WHOLE BLOOD USING QUANTITATIVE REAL-TIME PCR: A POTENTIAL DIAGNOSTIC/PROGNOSTIC ASSAY FOR ATHEROSCLEROSIS</td>
<td>50</td>
</tr>
<tr>
<td>Introduction</td>
<td>50</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>55</td>
</tr>
<tr>
<td>Results</td>
<td>59</td>
</tr>
<tr>
<td>Discussion</td>
<td>72</td>
</tr>
<tr>
<td>CHAPTER 4: MATHEMATICAL ANALYSIS OF THE ADHESION OF LIGAND CONJUGATED PARTICLES TO AN ATHEROSCLEROTIC PLAQUE</td>
<td>77</td>
</tr>
<tr>
<td>Introduction</td>
<td>77</td>
</tr>
<tr>
<td>Mathematical Model</td>
<td>80</td>
</tr>
</tbody>
</table>
SOLUTION STRATEGY .............................................................................................................................. 87
RESULTS................................................................................................................................................... 91
DISCUSSION .............................................................................................................................................. 98

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES .............................................................. 101
REFERENCES ............................................................................................................................................... 106

APPENDIX I: NOMENCLATURE AND ABBREVIATIONS .......................................................... 128

APPENDIX II: MATLAB CODE TO CALCULATE CONVERSION AT DIFFERENT STENOSIS HEIGHTS .......................................................... 132

APPENDIX III: REPRINT PERMISSIONS FOR THE MATERIAL USED IN THIS DISSERTATION .......................................................... 136
LIST OF TABLES

Table 2.1: Selectivity and ligand efficiency for LEAPs in different models of inflammation .................................................................35

Table 3.1: Slopes and correlation coefficients for standard curves in blood spiking studies..................................................................................69

Table 4.1: Values of the parameters used in the mathematical simulation.................90
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Schematic of atherosclerosis development and progression. Adapted by permission from McMillan Publishers Ltd: [Nature] (Libby 2002), Copyright (2002).</td>
<td>16</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic of proposed particle targeting mechanism.</td>
<td>19</td>
</tr>
<tr>
<td>1.3</td>
<td>Mechanisms of detachment of endothelial cells. “Reprinted from Clin Chim Acta, (Erdbruegger et al. 2006), Copyright (2006), with permission from Elsevier”</td>
<td>22</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic of ligand (α-VCAM-1 or IgG) conjugated particles.</td>
<td>29</td>
</tr>
<tr>
<td>2.2</td>
<td>Detection of ligand on the surface of polystyrene and PSA-PEG particles using pseudo-ELISA.</td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td>α-VCAM-1 polystyrene particles exhibit enhanced adhesion to ApoE^{-/-} mouse aorta in vivo.</td>
<td>34</td>
</tr>
<tr>
<td>2.4</td>
<td>α-VCAM-1 PSA-PEG particles exhibit enhanced adhesion to ApoE^{-/-} mouse aorta in vivo.</td>
<td>37</td>
</tr>
<tr>
<td>2.5</td>
<td>α-VCAM-1-polystyrene particles localized to plaque regions of ApoE^{-/-} mouse aorta.</td>
<td>40</td>
</tr>
<tr>
<td>2.6</td>
<td>Distribution of α-VCAM-1 polystyrene particles along the length of ApoE^{-/-} mouse aorta.</td>
<td>41</td>
</tr>
<tr>
<td>2.7</td>
<td>Adhesion of polystyrene particles to femoral artery endothelium.</td>
<td>44</td>
</tr>
<tr>
<td>3.1</td>
<td>Experimental steps in the determination of VCAM-1 and MCAM mRNA expression.</td>
<td>58</td>
</tr>
<tr>
<td>3.2</td>
<td>MCAM has a higher basal expression than VCAM-1 in HUVEC.</td>
<td>60</td>
</tr>
<tr>
<td>3.3</td>
<td>VCAM-1 is highly responsive to cytokine treatment whereas MCAM is not.</td>
<td>61</td>
</tr>
<tr>
<td>3.4</td>
<td>Incubation time for cytokine treated HUVEC affects VCAM-1 expression but not MCAM expression.</td>
<td>64</td>
</tr>
</tbody>
</table>
Figure 3.5: Standard curves for relative expression of VCAM-1 and MCAM in HUVEC spiked blood. ........................................................................................................ 67

Figure 3.6: Endothelial VCAM-1 to MCAM Ratio (EVMR) plots for whole blood spiked with HUVEC. ........................................................................................................ 68

Figure 3.7: Comparison of Endothelial Cell Activation Index (ECAI) for HUVEC and HUVEC spiked blood. .......................................................................................... 71

Figure 4.1: Schematic of plaque bearing vessel. An atherosclerotic plaque is modeled as a bell shaped constriction. .............................................................................. 82

Figure 4.2: Finite difference scheme for the solution of convective–diffusive transport equation. ............................................................................................................. 89

Figure 4.3: Velocity profiles in a plaque bearing aorta. ................................................................. 92

Figure 4.4: Increase in stenosis height causes an increase in conversion for particle adhesion within the plaque region. ............................................................... 94

Figure 4.5: Typical concentration profiles within the plaque region of the aorta......... 95

Figure 4.6: Conversion for particle adhesion increases with an increase in Damkohler number and decreases with an increase in Peclet number......... 97
CHAPTER 1: ATHEROSCLEROSIS AND MOLECULAR MARKERS FOR THE DEVELOPMENT OF NOVEL TERAPEUTICS AND DIAGNOSTICS

Atherosclerosis is a complex and common disease of the arteries which is a major cause of cardiovascular disease (CVD) and stroke worldwide (Anand et al. 2000; Lusis 2000; Pearson et al. 2003). In 2006, in the United States alone, CVD (which includes high blood pressure, coronary heart disease, and stroke) accounted for 34.3% of total deaths (Lloyd-Jones et al. 2009). Considering this high percentage of deaths due to CVD, where atherosclerosis is the underlying cause, it is imperative that the current diagnostic and therapeutic techniques are improved to more effectively detect and cure these diseases.

Atherosclerosis and its role in severe cardiovascular events

Continuing research within the last few decades has substantially improved our understanding of the processes underlying atherosclerosis. It has emerged that atherosclerosis is not just a bland deposition of lipids but involves complex physiological, biochemical and cellular processes (Lusis 2000; Ross 1999). One aspect of these processes is the inflammatory response of the cells within the vessel wall and immune cells of the blood. This inflammatory response generally follows the lipid deposition and involves recruitment of immune cells to the site of lipid deposition by the endothelial cells lining the vessel wall (Libby 2002; Lusis 2000; Ross 1999). Lipid deposition usually starts on the luminal vascular sites that are prone to developing atherosclerosis which
include curvatures and branches of the vessel (Fox et al. 1982; Nakashima et al. 1994; Nakashima et al. 1998). These sites experience a blood flow pattern that is substantially different from that experienced by the rest of the arterial network (Asakura and Karino 1990; Caro et al. 1969; VanderLaan et al. 2004). Macrophages start accumulating at atherosclerosis prone sites, engorge cholesterol and become foam cells (Libby 2002; Lusis 2000; Ross 1999). These foam cells populate the sub-endothelial regions of the arteries which start to form the initial lesions called fatty streaks (Lusis 2000; Ross 1999). Fatty streaks are not clinically significant structures as they do not cause any substantial clinical adverse events. But these fatty streaks are a first step in the complexities that follow over a longer period of a patient’s lifespan.

Fatty streaks serve as a precursor or seeding grounds for the formation of more advanced legions called plaques. Plaques present a more complex and challenging environment in the arterial lumen. In advanced stages of atherosclerosis, plaques can lead to a substantial loss of arterial elasticity and narrowing of the arterial lumen (Grey et al. 2003; van Popele et al. 2001). Fig. 1.1 (Adapted from Libby (2002)) depicts the gradual process of atherosclerosis development where a normal, healthy artery with a uniformly circular arterial lumen is shown. The lumen of this artery starts losing its structural uniformity and becomes occluded with plaque. Such an occlusion, when clinically significant, can severely restrict blood flow affecting normal functioning of the organs and tissues downstream. Also, as depicted in Fig. 1.1, a more severe clinical event that can occur is the sudden loss of plaque integrity leading to plaque rupture. This process releases a significant amount of cellular debris, fibrous material and other plaque
components into the blood stream causing clot formation which can get lodged into smaller arteries potentially causing myocardial infarction or stroke leading to death in many cases (Jackson 2007; Keenan et al. 2009; Langer 1991; Lassila 1993; Lusis 2000; Ogata et al. 1987; Rail 1984; Rudd et al. 2005; Schoenhagen et al. 2002).

Role of VCAM-1 in atherosclerosis

Inflammation in atherosclerosis plays a major role in causing the cardiovascular events discussed above (Libby 2002; Ross 1999). Endothelial cells lining the blood vessel lumen are actively involved in orchestrating the inflammatory processes at atherosclerosis prone vascular sites (Hansson 2001; Ross 1999). It has been observed that the arterial endothelium expresses various adhesion molecules at the onset of atherosclerosis at higher levels compared to those expressed by normal endothelium (Chettab et al. 2002; Quehenberger 2005). These adhesion molecules include P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Blankenberg et al. 2003; Cybulsky et al. 2001; Huo and Ley 2001; Nakashima et al. 1998). The presence of adhesion molecules leads to a chain of events causing leukocyte arrest and transmigration across the endothelium via what is called the leukocyte adhesion cascade (Gumina et al. 1997; Ley et al. 2007; Steeber et al. 2005). This process leads to further complications of the disease as discussed above.
Figure 1.1: Schematic of atherosclerosis development and progression. Adapted by permission from McMillan Publishers Ltd: [Nature] (Libby 2002), Copyright (2002).
Accumulating evidence suggests that VCAM-1 is increased at the sites of atherosclerosis, and is known to contribute greatly in the tethering and firm adhesion events of leukocytes in atherosclerosis (Cybulsky et al. 2001; Cybulsky et al. 1999; Ley and Huo 2001; Nakashima et al. 1998; O'Brien et al. 1993). VCAM-1, a member of the immunoglobulin gene superfamily, exhibits alternative splicing into two isoforms (Cybulsky et al. 1991). Studies have indicated that monocyte adhesion and trans-endothelial migration occurs through interactions between these two isoforms of VCAM-1 (expressed by endothelial cells) and VLA-4 (the integrin expressed by monocytes) (Chuluyan et al. 1995; Cybulsky et al. 2001). Monocyte adhesion and migration across the vascular endothelium is one of the major steps in atherosclerosis that leads to plaque formation and other complications discussed above.

**Site specific targeting in atherosclerosis**

The concept of site specific targeting using molecules expressed on the surface of inflamed cells mimics leukocyte adhesion to sites of inflammation. It has opened new avenues in developing innovative diagnostics and therapeutics. Extensive biomedical research in the last few decades, especially in the area of cell adhesion, has enabled the development of molecular targets that are specific to the disease and also to the site where the disease is more active (Blankenberg et al. 2003; Cockerill et al. 2001; Danese et al. 2005; Gueguez et al. 2007; Gulubova et al. 2006; Huo and Ley 2001; Issekutz and Issekutz 2002; Issekutz and Issekutz 1991; Kunkel and Ley 1996; Ley 2003; Ley et al. 1991; Ley and Huo 2001; Nakashima et al. 1998; Pritchard et al. 1995; Springer 1994;
Steeber et al. 2005). Various inflammatory diseases are characterized by unique molecular signatures (Danese et al. 2005; Hwang et al. 1997; Issekutz and Issekutz 2002; Nakashima et al. 1998; Pritchard et al. 1995). These molecular signatures can be defined by one or more molecules that play a major role in the initiation, development or progression of the disease under consideration (Chuluyan et al. 1995; Cybulsky et al. 2001; Guezguez et al. 2007; Kunkel and Ley 1996; Swirski et al. 2006). Thus, a site specific targeting system can be tailor made for each inflammatory disease that presents a unique molecular signature. Atherosclerosis is also an inflammatory disease characterized by elevated expression of VCAM-1 as discussed above. Thus, a site specific targeting system may be developed for atherosclerosis using endothelial VCAM-1 as the target molecule.

A variety of approaches have been proposed to target specific agents to sites of atherosclerosis. These agents can range from simple molecular moieties to more complex particle based agents. Examples include peptides and peptide moieties (Burtea et al. 2008; Hong et al. 2008; Kelly et al. 2006), antibodies (McCarron et al. 2005; Tsimikas and Shaw 2002), liposome and particle based moieties (Kelly et al. 2005; Lestini et al. 2002; Nahrendorf et al. 2006; Scott et al. 2007). In this dissertation, a polymeric particle based targeting system was developed which is presented in Chapter 2. Such a targeting system can specifically target atherosclerotic sites as shown in the schematic in Fig. 1.2. Thus, these and other similar polymeric particle based targeting systems can be used in delivering therapeutics to sites of atherosclerosis and may easily be adapted in devising imaging modalities. Indeed, studies have shown that the targeting systems similar to the
one developed in this doctoral work can work in imaging applications (Ferrante et al. 2009; Kelly et al. 2005).

Figure 1.2: Schematic of proposed particle targeting mechanism.

Although such imaging modalities can serve as excellent diagnostic tools in assessing the extent of atherosclerosis, they would need to meet stringent regulations when used in a clinical setting since they involve injecting foreign material into humans. By contrast, a blood based diagnostic approach need not meet stricter regulations since no foreign material is introduced and only whole blood is drawn from patients for analysis. Thus, a blood based diagnostic approach would be very attractive compared to a diagnostic approach based on injectable imaging agents.
Blood based diagnostics for atherosclerosis

Blood based diagnostic techniques are conceptually simple and primitive assays exist for detecting the presence of atherosclerosis using markers of inflammation. A number of inflammatory markers have been identified for this purpose. Examples include C-reactive protein (CRP), cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), and soluble adhesion molecules such as sICAM-1, sVCAM-1, sP-selectin and sE-selectin (Erren et al. 1999; Eschen et al. 2005; Hwang et al. 1997; Libby and Ridker 1999; Magyar et al. 2003; Ridker et al. 1998). Since many of these markers are implicated in a range of inflammatory diseases, it is difficult to correlate their levels with atherosclerosis development unless a combination method is used where levels of these markers are coupled with other indications of atherosclerosis. This motivates the development of a diagnostic technique that utilizes more than one aspect of atherosclerosis, e.g. the number of endothelial cells and their activation state, may improve our ability to detect atherosclerosis early in its development so that adverse clinical events may be avoided.

Recent studies in atherosclerosis have indicated that the endothelium in the vascular segments affected by atherosclerosis is subject to wear and tear due to hydrodynamic and biochemical processes (Davignon and Ganz 2004; Makin et al. 2004; Zhang 2008). This led to the hypothesis that such wear and tear would cause substantial vascular damage and an increase in circulating endothelial cells (CECs). Fig. 1.3 schematically represents a typical vascular damage site which shows three major mechanisms viz., necrosis, apoptosis and defective adhesion. The defects in the
endothelial cell adhesive mechanisms (e.g. the reduced affinity of the integrins) are caused by the action from cytokines, mechanical forces or nitric oxide (NO) (Erdbruegger et al. 2006). These mechanisms are responsible for endothelial cell detachment.

The mechanisms shown in Fig. 1.3 are also implicated in atherosclerosis as discussed in the beginning of this chapter and hence may lead to similar vascular damage in atherosclerosis. Endothelial cells at the sites of atherosclerosis experience biochemical insults in addition to mechanical forces. Hence the detached endothelial cells may carry a unique molecular signature. The most obvious molecular candidate would be VCAM-1 since it is over-expressed at sites of atherosclerosis as discussed in previous paragraphs. Thus, development of a blood based assay that can determine the nature and number of CECs in blood samples from atherosclerotic patients using a marker such as VCAM-1 could serve as an excellent diagnostic tool.

Chapter 3 presents a study that uses quantitative real-time PCR (qRTPCR) that is the first step towards the development of an assay to detect endothelial cells from atherosclerotic patient’s whole blood samples. This technique uses 5’-3’ exonuclease activity of a DNA polymerase enzyme (Holland et al. 1991). qRTPCR is a very sensitive technique that can efficiently detect the presence of an mRNA transcript even if only a few copies are present. (Gao et al. 2008; Gibson et al. 1996; Ginzinger 2002; Heid et al. 1996).
Figure 1.3: Mechanisms of detachment of endothelial cells. “Reprinted from Clin Chim Acta, (Erdbruegger et al. 2006), Copyright (2006), with permission from Elsevier”

“-” indicates defective adhesive properties of the endothelial cells caused by cytokines, mechanical forces and nitric oxide (NO).
The overall objective of this doctoral study is to develop novel therapeutic and diagnostic approaches for atherosclerosis using inflammatory markers.

Objective I: In Chapter 2, a biodegradable particle based targeting system is presented that utilizes the over-expression of VCAM-1, an inflammatory marker of atherosclerosis. The studies show for the first time that such a targeting system is capable of achieving site specific adhesion in an atherosclerotic mouse model. The adhesion of ligand conjugated particles within the plaque regions is further characterized and it is shown that the adhesion within the plaque regions follows a profile that seems to be unique to atherosclerosis.

Objective II: The mechanism of plaque development and progression leaves unique molecular and cellular signatures in its surroundings. In Chapter 3, it is shown that these molecular (VCAM-1) and cellular (circulating endothelial cells) signatures could be exploited in devising a real-time PCR based technique that may serve as a diagnostic tool in determining the extent of vascular damage in atherosclerosis.

Objective III: The unique particle adhesion profiles within the plaque regions observed in Chapter 2 inspired the development of a mathematical model for particle adhesion to plaques. The resulting mathematical model is presented in Chapter 4 where certain key parameters intrinsic to atherosclerosis are analyzed for their effect on particle adhesion to a plaque.
CHAPTER 2: POLYMERIC PARTICLES CONJUGATED WITH A LIGAND TO VCAM-1 EXHIBIT SELECTIVE, AVID AND FOCAL ADHESION TO SITES OF Atherosclerosis

Introduction

Targeted drug delivery via the vascular endothelium holds great promise for significantly improving therapeutics for a host of inflammatory diseases. This targeting approach is based on the fact that certain endothelial cell adhesion molecules (ECAMs) [e.g. E-selectin, ICAM-1, VCAM-1 and P-selectin (Ley et al. 2007; Luscinskas and Gimbrone 1996; McEver 2001; Springer 1994)] are over-expressed at sites of inflammation. Drug carriers conjugated with biomolecules that can adhere to one or more of the ECAMs could be used to specifically target therapeutic agents to sites of inflammation. The composition of the drug carriers could be tailored to suit the design needs using a variety of materials, including lipids and biodegradable polymers (Eniola and Hammer 2005; Eniola et al. 2002; Muro et al. 2006a; Muro et al. 2006b; Sakhalkar et al. 2003; Sakhalkar et al. 2005; Scott et al. 2007; Spragg et al. 1997; Stahn et al. 2001). Biodegradable polymers are receiving increased interest due to their many advantages which are summarized in a previously published study (Sakhalkar et al. 2003; Soppimath et al. 2001).

Some of the studies in the Goetz laboratory have previously provided several pieces of evidence which suggest that targeting of biodegradable particles to sites of inflammation via ECAMs is feasible. One such study found that biodegradable polymeric (PLA-PEG) particles conjugated with ligands to E-selectin, VCAM-1, or ICAM-1 exhibited enhanced adhesion to endothelial cells in an *in vitro* inflammation model (Sakhalkar et al. 2003). This study also showed that PLA-PEG particles conjugated with ligands to E-selectin or P-selectin exhibit enhanced adhesion to inflamed endothelium in a TNF-α or tissue injury model, respectively, of inflammation *in vivo*. Finally, another study showed that particles conjugated with a ligand to VCAM-1 exhibit enhanced adhesion to sites of colonic inflammation in DSS-dextran murine model of colitis (Sakhalkar et al. 2005).

Although the *in vivo* studies done to date suggest this approach will work in a variety of pathological inflammation settings, it is important to note that they all involved adhesion in the microcirculation (i.e. the post-capillary venules). One of the more important diseases of inflammation is atherosclerosis which involves the larger vessels (e.g. the aorta). The anatomy of the microcirculation is strikingly different from that of the aorta (e.g. the location relative to the heart, the diameter and branching patterns of the vessels) and the differences in the vascular architecture give rise to dramatically different fluid dynamics (e.g. inertial forces dominate in the aorta and viscous forces dominate in the microcirculation) (Goldsmith and Turitto 1986). At a fundamental level, adhesion is an issue of reaction kinetics, mass transfer and a force balance all of which are intertwined with the operative fluid dynamics. Thus, it is unclear if results obtained in
the microcirculation can be extrapolated to that which will occur in the larger vessels. In this study, the Leukocyte-endothelial cell adhesive particle (LEAP) targeting approach was explored for its effectiveness in atherosclerosis which is the disease of large blood vessels.

As discussed in Chapter 1, atherosclerosis is characterized by lipid accumulation and follows a complex physiological and molecular pathway of plaque development (Libby et al. 2002; Lusis 2000; Ross 1999). A key mechanism of atherogenesis is the adhesion of T-lymphocytes and monocytes to the vascular endothelium which is mediated, in part, by ECAMs (Dong and Wagner 1998; Galkina and Ley 2007; Libby et al. 2002; Lusis 2000; Ross 1999). Several studies have shown that the expression of VCAM-1, described as an athero-ELAM (Cybulsky and Gimbrone 1991), is increased at sites of atherosclerosis. Indeed, VCAM-1 has been shown to be up-regulated on aortic endothelium of Apolipoprotein E deficient (ApoE^{-/-}) mice kept on a high fat diet (Nakashima et al. 1998). Thus, this study explored the hypothesis that polymeric particles conjugated with a ligand to VCAM-1 would exhibit enhanced adhesion at sites of atherosclerosis in the ApoE^{-/-} murine model.
Materials and Methods

Materials

Some of the materials used in this study were the same as the materials used in previous studies in the Goetz laboratory and purchased from the same vendors and can be found in previous papers (Sakhalkar et al. 2003; Sakhalkar et al. 2005). 2 μm streptavidin coated fluoresbrite YG carboxylated microspheres were from Polysciences Inc. (Warrington, PA). O-phenylenediamine (OPD), Oil Red O (1-([4-(Xylylazo)xylyl]azo)-2-naphthol) was from Sigma Aldrich (St. Louis, MO). Formaldehyde was from Fisher Scientific (Fair Lawn, NJ). Avertin (a combination of 2,2,2-tribromoethanol, tertiary amylalcohol and saline from Sigma Aldrich) was prepared and supplied by Dr. Ramiro Malgor our collaborator at Ohio University. ApoE−/− (B6.129P2-Apoem1Unc/J) and C57BL/6J female mice were from Jackson Laboratories (Bar Harbor, Maine). C57BL/6J mice were fed a normal diet and at 5 weeks of age ApoE−/− mice were put on a high cholesterol diet (Harlan Teklad, Madison, WI). 25-35 week old mice were used for the experiments.

Synthesis of PSA-PEG-biotin and preparation of particles

Biotinylated PSA-PEG biodegradable particles were synthesized and supplied by our collaborator, Dr. Justin Hanes, at the Johns Hopkins University. The particles used in this study have an average size of 1.7±0.03μm. The details of the particle synthesis and key features can be found elsewhere (Deosarkar et al. 2008; Fu et al. 2002).
**Ligand conjugation and detection**

Biotin-avidin chemistry was used to conjugate ligands to both the polystyrene and the PSA-PEG particles. Ligand conjugated particles are shown in Fig. 2.1 where biotin-avidin chemistry is schematically represented. For polystyrene particles, a one step conjugation was used. In brief, 2 µm yellow-green streptavidin coated polystyrene particles were washed in PBS with 1% BSA and incubated (4°C, 30 minutes) with biotinylated α-VCAM-1 or IgG diluted in PBS (15 µg/ml). Subsequently, the ligand-conjugated particles were washed in PBS, 1% BSA and re-suspended in PBS to the final working concentration. Ligand conjugation to PSA-PEG biodegradable particles was achieved in a two-step conjugation process similar to that described previously (Sakhalkar et al. 2003; Sakhalkar et al. 2005). A modified ELISA technique (Dickerson et al. 2001; Sakhalkar et al. 2005) was used to detect the presence of ligand on the polystyrene and PSA-PEG particles. Optical density at 450 nm (O.D. 450nm), which correlates with the amount of ligand present on the particles, was recorded.

**In vivo adhesion assay**

The technique used to analyze particle adhesion to the aorta was a modification and extension of techniques previously used to study particle adhesion in vivo (Burch et al. 2002; Sakhalkar et al. 2003; Sakhalkar et al. 2005). Ligand conjugated fluorescent particles (1.6 x 10^7 in 200 µl PBS) were injected into the cannulated jugular vein in a bolus fashion. Note that the femoral artery was exteriorized prior to injection and observed under fluorescent microscopy after injection to verify that particles had entered
the blood stream. The interactions of the particles with the femoral artery endothelium were recorded on a video recording tape by moving from one end of the artery to the other (5 to 10 fields of views). Approximately 15 minutes after the injection of the particles, blood was removed via exsanguination and the vasculature perfused (PBS for 10 minutes followed by 10% formalin for 10 minutes) through the left ventricle using a syringe pump. Subsequently, the aorta was removed, cleaned, cut open longitudinally, and mounted en face between a glass slide and a cover-slip.

![Diagram of ligand conjugated particles.](image)

**Figure 2.1:** Schematic of ligand ($\alpha$-VCAM-1 or IgG) conjugated particles.

The aortic preparation was transferred to the stage of a microscope and the number of particles adherent in sequential fields of view along the entire length of the aorta (i.e. the ascending aorta, aortic arch, the descending aorta and the abdominal aorta) was determined. These numbers were normalized to the area of the field of view, and averaged. This average is the result for a single mouse. In certain experiments, Oil-Red-
O (a dye that stains lipids) was used to stain the plaques on the aorta harvested from ApoE<sup>−/−</sup> mice previously injected with α-VCAM-1 polystyrene particles. In these experiments, image analysis software (Image J 1.35v, NIH) was used to analyze 13 different images. For each image, image analysis was used to estimate the total area of the plaque regions and the total area of the non-plaque regions. Additionally, each plaque was divided into a central region and a periphery region by drawing a contour 46 ± 9 µm, the approximate length scale of 1-2 aortic endothelial cells (Kevil and Bullard 2001), inside the plaque boundary. The periphery region was defined as that which lay outside the contour and the central region was defined as that which lay inside the contour. The total area of the central and periphery regions were also determined using image analysis. The number of particles adherent in each of these regions (i.e. plaque, non-plaque, central and periphery) were determined and normalized to the area under observation. The results are presented as average of particles from 13 different images.

**Statistics**

A student’s t-test was used for comparing two means. In cases involving two factors, i.e. mouse type and ligand, a two-factor ANOVA along with a Tukey-Kramer multiple comparison test were used. ANOVA and Tukey-Kramer tests were performed using NCSS 2004 statistical analysis software (Kaysville, UT). p<0.05 were considered significant.
Results

$\alpha$-VCAM-1 polystyrene particles exhibit enhanced adhesion to the aorta of ApoE$^{-/-}$ mice relative to wild type mice and polystyrene particles conjugated with IgG in an *in vivo* adhesion assay

Initial studies were conducted with polystyrene particles due to their ease of handling and detection. The ApoE$^{-/-}$ mouse is a well accepted model of human atherosclerosis (Nakashima et al. 1994) in which VCAM-1 is upregulated at the sites of atherosclerosis (Iiyama et al. 1999; Nakashima et al. 1998). Thus, the adhesion of $\alpha$-VCAM-1 polystyrene particles was first investigated in the ApoE$^{-/-}$ mouse. Wild type mice and particles conjugated with IgG were used as controls.

As shown in Fig. 2.2A, a biotinylated VCAM-1 antibody ($\alpha$-VCAM-1) was successfully conjugated to streptavidin conjugated polystyrene particles. Similarly, biotinylated IgG (a negative control ligand) was also successfully conjugated to the particles (Fig. 2.2A). Suspensions of the ligand-conjugated particles were injected into separate mice, the aortas harvested and the adhesion quantified by fluorescent microscopy. As shown in Fig. 2.3, $\alpha$-VCAM-1 polystyrene particles exhibited enhanced adhesion to ApoE$^{-/-}$ mouse aorta compared to the level of adhesion to wild type mouse aorta (Fig. 2.3A vs. 2.3B). Similarly, $\alpha$-VCAM-1 polystyrene particles exhibited greater adhesion to ApoE$^{-/-}$ mouse aorta compared to the level of adhesion of IgG particles to ApoE$^{+/+}$ mouse aorta (Fig. 2.3A vs. 2.3C).
Figure 2.2: Detection of ligand on the surface of polystyrene and PSA-PEG particles using pseudo-ELISA.

α-VCAM-1 and IgG can be conjugated to (A) polystyrene particles and (B) PSA-PEG particles. Particles were conjugated with the ligand shown. Bound ligand was detected via an HRP-secondary antibody and OPD (o-phenylenediamine). Optical density (O.D.) at 450nm correlates with the amount of ligand on the particle. One-way ANOVA indicated a difference in the means (p<0.05). * significantly different from other 2 bars (p<0.05).
A two-factor ANOVA indicated that there was a significant effect of mouse type (i.e. ApoE\(^{-/-}\) vs. wild type) and ligand type (i.e. \(\alpha\)-VCAM-1 vs. IgG) on the adhesion of the polystyrene particles (\(p<0.05\)). A post-hoc, multi-comparison Tukey-Kramer test indicated that the adhesion of \(\alpha\)-VCAM-1 conjugated particles to ApoE\(^{-/-}\) mouse aorta was significantly higher than (a) the adhesion of IgG particles to ApoE\(^{-/-}\) mouse aorta and (b) the adhesion to wild type mouse aorta (Fig. 2.3E).

As described and defined in previous studies in Goetz laboratory (Sakhalkar et al. 2003; Sakhalkar et al. 2005), two key parameters, i.e. ligand efficiency and selectivity, can be used to characterize the effectiveness of the targeting. In the context of the present study, ligand efficiency is the ratio of the number of \(\alpha\)-VCAM-1 particles relative to the number of IgG particles that bind to ApoE\(^{-/-}\) mouse aorta and selectivity is the ratio of the number of \(\alpha\)-VCAM-1 particles that bind to ApoE\(^{-/-}\) mouse aorta relative to the number of \(\alpha\)-VCAM-1 particles that bind to wild type mouse aorta. For the polystyrene particles, the ligand efficiency was \(~32\) and the selectivity was \(~2\) (Table 2.1).
Figure 2.3: α-VCAM-1 polystyrene particles exhibit enhanced adhesion to ApoE⁻/⁻ mouse aorta *in vivo*.

(A) α-VCAM-1 particles exhibit significant adhesion to ApoE⁻/⁻ mouse aorta. (B) α-VCAM-1 particles exhibit limited adhesion to wild type mouse aorta. (C) IgG particles show little adhesion to ApoE⁻/⁻ mouse aorta. (D) IgG particles exhibit negligible adhesion to wild type mouse aorta. (E) Bar chart of results. Error bars indicate SEM, n=3. Two-factor ANOVA indicated that each factor has an individual effect as well as a combined effect on the adhesion (p<0.05). * indicates significantly different from other 3 bars (p<0.05).
Table 2.1

Selectivity and ligand efficiency for LEAPs in different models of inflammation

<table>
<thead>
<tr>
<th>Inflammation Model</th>
<th>In vitro/In vivo</th>
<th>Polymer</th>
<th>Ligand</th>
<th>Selectivity</th>
<th>Ligand Efficiency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE−/−</td>
<td>In vivo</td>
<td>Polystyrene</td>
<td>α-VCAM-1</td>
<td>2</td>
<td>32</td>
<td>Present study</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>In vivo</td>
<td>PSA-PEG</td>
<td>α-VCAM-1</td>
<td>5</td>
<td>16</td>
<td>Present study</td>
</tr>
<tr>
<td>Colitis</td>
<td>In vivo</td>
<td>Polystyrene</td>
<td>α-VCAM-1</td>
<td>3</td>
<td>8</td>
<td>(Sakhalkar et al. 2005)</td>
</tr>
<tr>
<td>Colitis</td>
<td>In vivo</td>
<td>PLA-PEG</td>
<td>α-VCAM-1</td>
<td>3</td>
<td>13</td>
<td>(Sakhalkar et al. 2005)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>In vivo</td>
<td>PLA-PEG</td>
<td>α-E-selectin</td>
<td>6</td>
<td>5</td>
<td>(Sakhalkar et al. 2003)</td>
</tr>
<tr>
<td>Trauma</td>
<td>In vivo</td>
<td>PLA-PEG</td>
<td>PSGL-1 (19.ek.Fc)</td>
<td>-</td>
<td>10</td>
<td>(Sakhalkar et al. 2003)</td>
</tr>
<tr>
<td>No inflammation</td>
<td>In vivo</td>
<td>Polystyrene</td>
<td>α-ICAM-1</td>
<td>-</td>
<td>9*</td>
<td>(Muro et al. 2006a)</td>
</tr>
<tr>
<td>No inflammation</td>
<td>In vivo</td>
<td>PLGA</td>
<td>α-ICAM-1</td>
<td>-</td>
<td>9*</td>
<td>(Muro et al. 2006a)</td>
</tr>
<tr>
<td>HUVEC</td>
<td>In vitro</td>
<td>PLA-PEG</td>
<td>α-VCAM-1</td>
<td>15</td>
<td>33</td>
<td>(Sakhalkar et al. 2003)</td>
</tr>
<tr>
<td>HUVEC</td>
<td>In vitro</td>
<td>PLA-PEG</td>
<td>α-E-selectin</td>
<td>12</td>
<td>27</td>
<td>(Sakhalkar et al. 2003)</td>
</tr>
<tr>
<td>HUVEC</td>
<td>In vitro</td>
<td>Polystyrene</td>
<td>α-ICAM-1</td>
<td>-</td>
<td>45†</td>
<td>(Muro et al. 2006a)</td>
</tr>
</tbody>
</table>

*indicates that the value was calculated from the results presented in the corresponding publication.

†These values were calculated by taking a ratio of the biodistribution data given in Muro et al. 2006a
α-VCAM-1 PSA-PEG biodegradable particles exhibit enhanced adhesion to the aorta of ApoE⁻/⁻ mice relative to wild type mice and PSA-PEG particles conjugated with IgG in an in vivo adhesion assay

The results presented in Fig. 2.3 suggest that polymeric particles conjugated with α-VCAM-1 will exhibit enhanced adhesion to sites of atherosclerosis. Most relevant to drug delivery are particles made from biodegradable polymers. Recently, a study described the synthesis of a biodegradable block copolymer composed of poly(sebacic acid) and biotinylated polyethylene glycol (PSA-PEG-biotin) (Hanes and Fu 2006). Particles made from PSA-PEG-biotin have several salient features which are summarized in previously published studies (Fu et al. 2004; Fu et al. 2002; Gref et al. 1994). PSA-PEG particles, obtained from Hanes laboratory at Johns Hopkins University, were conjugated with α-VCAM-1 and used for investigating their adhesion to atherosclerotic tissue in the ApoE⁻/⁻ mouse using an assay identical to the one used above for polystyrene particles.

α-VCAM-1 and IgG were successfully coupled, via biotin-avidin chemistry, to porous particles made from PSA-PEG (Fig. 2.2B). As shown in Fig. 2.4, α-VCAM-1 PSA-PEG particles exhibited enhanced adhesion to ApoE⁻/⁻ mouse aorta compared to the level of adhesion to wild type aorta (Fig. 2.4A vs. 2.4B). α-VCAM-1 PSA-PEG particles exhibited greater adhesion to ApoE⁻/⁻ mouse aorta compared to the level of adhesion of IgG particles to ApoE⁻/⁻ mouse aorta (Fig. 2.4A vs. 2.4C).
Figure 2.4: α-VCAM-1 PSA-PEG particles exhibit enhanced adhesion to ApoE<sup>-/-</sup> mouse aorta <i>in vivo</i>.

(A) α-VCAM-1 PSA-PEG particles exhibit significant adhesion to ApoE<sup>-/-</sup> mouse aorta. (B) α-VCAM-1 PSA-PEG particles exhibit limited adhesion to wild type mouse aorta. (C) IgG PSA-PEG particles show little adhesion to ApoE<sup>-/-</sup> mouse aorta. (D) IgG PSA-PEG particles exhibit negligible adhesion to wild type mouse aorta. (E) Bar chart of results. Error bars indicate SEM, n=3. Two-factor ANOVA indicated that each factor has an individual effect as well as a combined effect on the adhesion (p<0.05). * indicates significantly different from other 3 bars (p<0.05).
A two-factor ANOVA indicated that there was a significant effect of mouse type (i.e. ApoE\(^{-/-}\) vs. wild type) and ligand type (i.e. \(\alpha\)-VCAM-1 vs. IgG) on the adhesion of the PSA-PEG particles (p<0.05). A post-hoc, multi-comparison Tukey-Kramer test indicated that the adhesion of \(\alpha\)-VCAM-1 conjugated particles to ApoE\(^{-/-}\) mouse aorta was significantly higher than (a) the adhesion of IgG particles to ApoE\(^{-/-}\) mouse aorta and (b) the adhesion to wild type mouse aorta (Fig. 2.4E). For the PSA-PEG particles, the ligand efficiency was ~16 and selectivity was ~5 (see Table 2.1).

**\(\alpha\)-VCAM-1 particles exhibit preferential adhesion to plaque regions within ApoE\(^{-/-}\) mouse aortas**

The results of the adhesion studies for both polystyrene and PSA-PEG particles described in the previous sections indicated focal adhesion of the particles within the aorta (see for example Figures 2.3A and 2.4A). Indeed, a detailed analysis of the images suggested that the \(\alpha\)-VCAM-1 particles exhibit selective adhesion to the plaque regions. To probe this conjecture, a set of adhesion assays were conducted with \(\alpha\)-VCAM-1 polystyrene particles and ApoE\(^{-/-}\) mice wherein at the end of the experiments, the luminal surface of the aorta was stained with Oil Red O. A representative image from this study is shown in Fig. 2.5A. The images reveal focal adhesion of \(\alpha\)-VCAM-1 conjugated particles (green fluorescent) to the plaque regions (stained red) of the ApoE\(^{-/-}\) mouse aorta. The level of adhesion of the \(\alpha\)-VCAM-1 particles to plaque and non-plaque regions was quantified and the results are shown in Fig. 2.5B. As can be seen, a significantly greater number (~2.5-fold) of \(\alpha\)-VCAM-1 particles bind to plaque regions compared to non-
plaque regions of the aorta. Within the plaque region, the level of adhesion of α-VCAM-1 particles to the periphery of the plaque was compared to the level of adhesion of α-VCAM-1 particles to the central region of the plaque. As shown in Fig. 2.5C, α-VCAM-1 particles exhibited significantly greater adhesion to the plaque periphery compared to the central region of the plaque.

The above results indicate that regions of the aorta bearing plaques would exhibit higher particle adhesion compared to the regions without plaques. To further validate this observation, the number of particles per field of view along the entire length of aorta were calculated and plotted as shown in Fig. 2.6. This was done to determine if there existed any pattern in the adhesion along the length of the aorta. Since the adhesion data was obtained by counting particles in each field of view starting from the aortic arch region to the iliac bifurcation region on the aorta, an interesting pattern was observed in the adhesion of α-VCAM-1 conjugated particles that roughly correlates with the atherosclerotic prone sites on the aorta as shown in Fig. 2.6. The data for adhesion of α-VCAM-1 particles in ApoE^{-/-} mice is presented here. Each condition was repeated thrice. Statistical significance may not be determined in this case because the data collected for three mice can not be matched in terms of exact location of the field of view. But this data suggests that the maximum adhesion of the particles was observed in the regions of aortic arch, the mid-abdominal region and also some enhanced adhesion in the region close to the iliac bifurcation whereas, minimal adhesion was observed to the rest of the aortic endothelium.
Figure 2.5: α-VCAM-1-polystyrene particles localized to plaque regions of ApoE−/− mouse aorta.

(A) A representative image showing adherent α-VCAM-1 particles (green spheres) on ApoE−/− mouse aorta. The plaques stained with Oil Red O appear red. The α-VCAM-1 particles appear to preferentially adhere at or near areas of plaque. Numbers on the image indicate separate areas used in the analysis. (B) The number of particles adherent per unit area on plaque and non-plaque regions was determined. (C) The number of particles adherent per unit area on the plaque periphery and the central region of the plaques were determined. *indicates significant difference from other bar (p<0.05).
Figure 2.6: Distribution of $\alpha$-VCAM-1 polystyrene particles along the length of ApoE$^{-/-}$ mouse aorta.

$\alpha$-VCAM-1 polystyrene particle adhesion was plotted as a function of position (field of view) along the aorta starting at the aortic arch and continuing to the iliac bifurcation. The adhesion appears to be non-random with distinct segments along the aorta having enhanced adhesion (represented by dotted arrows).
α-VCAM-1 particles exhibit a non-selective adhesion to the femoral artery

The femoral artery has been reported as one of the vascular parts vulnerable to atherosclerosis under advanced atherosclerotic conditions (Lisowska et al. 2005; Ross et al. 1984; Sontheimer 2006; Wood et al. 2006). Hence, the adhesion of ligand conjugated polymeric particles to femoral artery endothelium was quantified.

As described in the methods section, intravital microscopy was used for this study. Recorded videos of particle interactions with femoral artery were used to quantify particle adhesion to the femoral artery. PSA-PEG biodegradable particles were excluded from this analysis since they were not visible in the femoral artery videos, probably due to weak fluorescence intensity of the fluorophore on PSA-PEG particles. Hence, for studying particle adhesion to the femoral artery, only the polystyrene particle adhesion data was analyzed. Adherent particles were counted in each field of view, averaged and reported as particles attached per square mm of femoral arterial endothelium. These results were obtained for both ApoE-/- and wild type (C57BL/6J) mice with α-VCAM-1 and IgG as ligands on the particle surfaces. The results are shown in Fig. 2.7. It was found that particles with α-VCAM-1 as a ligand showed enhanced adhesion to femoral artery endothelium compared to IgG (a control ligand) conjugated particles, as can be seen from Fig. 2.7A-C. A ligand efficiency of ~7.8 was obtained in this case. When the data in Fig. 2.7C was analyzed using two-factor ANOVA a significant effect of ligand was observed (p=0.0025<0.05). In contrast, the adhesion of particles conjugated with α-VCAM-1 to femoral artery endothelium of ApoE-/- and C57BL/6J mice did not show any significant difference. This was also confirmed by ANOVA (p=0.987>0.05 for the
effect of type of mouse). A selectivity of ~1.1 was obtained in the case of particle adhesion to femoral artery endothelium.
Figure 2.7: Adhesion of polystyrene particles to femoral artery endothelium.

Images A and B are extracted from video recordings of ligand conjugated polystyrene particle interactions with femoral artery endothelium and serve as representative example of the results. A) α-VCAM-1 conjugated polystyrene particles show enhanced adhesion compared to B) IgG conjugated polystyrene particles to the endothelium of the femoral artery, C) α-VCAM-1 conjugated polystyrene particles show increased adhesion to femoral artery endothelium compared to a control ligand (IgG) but no significant difference compared to a control mouse. Two-factor ANOVA indicated that only type of ligand had an effect (p=0.0025<0.05) on the adhesion but not the type of mouse (p=0.987>0.05). * indicates significantly different from second and fourth bar but not third bar.
Discussion

Previously published studies in the area of site specific targeting has demonstrated that polymeric particles conjugated with ligands to inducible ECAMs exhibit avid and selective adhesion to inflamed microvasculature (Sakhalkar et al. 2003; Sakhalkar et al. 2005). In the present study, experiments were conducted to determine if LEAPs would exhibit similar behavior in atherosclerosis which is an inflammatory disease. VCAM-1 mAb was conjugated to polymeric particles (polystyrene and PSA-PEG) and the adhesion of the resulting ligand conjugated particles to the aorta of ApoE⁻/⁻ mice was investigated. It was found that the ligand-conjugated particles exhibit avid and selective adhesion to sites of atherosclerosis (Fig. 2.3 and 2.4). A detailed analysis of the adhesion revealed that within the ApoE⁻/⁻ mouse aorta, the particles exhibit focal adhesion to the plaque regions. Indeed, a 2.5-fold higher level of adhesion to plaque regions relative to non-plaque regions was observed (Fig. 2.5). Interestingly, the particles appeared to be highly concentrated at the periphery of the plaques, as opposed to the central region (see for example Figs. 2.3A, 2.4A and Fig. 2.5C). Such a result would be expected given the observation made by Cybulsky’s group (Iiyama et al. 1999) that VCAM-1 is most pronounced in the shoulder regions of the plaque. Combined, these results demonstrate that polymeric drug carriers made from biodegradable polymers such as PSA-PEG and conjugated with a ligand to VCAM-1, exhibit enhanced, selective and focal adhesion to sites of atherosclerosis.
The above studies extend the results of a previous in vivo study in the Goetz laboratory where it was observed that α-VCAM-1 PLA-PEG particles exhibit avid and selective adhesion to colitic microvasculature in a murine model of colitis (Sakhalkar et al. 2005). While it is difficult to compare adhesion in the microvasculature to adhesion in the aorta (e.g. the number of particles delivered to each vessel may differ dramatically), ligand efficiency and selectivity are ratios which, to a certain extent, mitigate this issue. In the present study, it was found that the ligand efficiency and selectivity was ~16 and ~5 for the α-VCAM-1 PSA-PEG particles in the aorta which is comparable to what was observed previously for α-VCAM-1 PLA-PEG particles in a murine model of colitis, i.e. ~13 and ~3 for ligand efficiency and selectivity respectively (Sakhalkar et al. 2005).

Above observations suggest that the LEAP targeting approach has similar effectiveness in small or large vessels. Given the differences in the fluid dynamics present in the aorta versus the post-capillary venules (Goldsmith and Turitto 1986) and the fact that numerous in vitro studies have demonstrated that cell and particle adhesion is a function of local fluid dynamics (Hammer et al. 1993; Shinde Patil et al. 2001; Tempelman et al. 1994), this is a somewhat surprising result which warrants further investigation. Additionally, analysis of the data from particle adhesion to the femoral artery indicated a selectivity of ~ 1.1 and a ligand efficiency of ~7.8 which suggests that α-VCAM-1 particles did not show a selective adhesion to femoral artery endothelium (Fig. 2.7) and VCAM-1 expression profiles in femoral artery may be different from that
in the aorta. Hence, a more detailed study is required to investigate the adhesion of ligand conjugated particles to different vascular beds. Overall, a systematic study of the effect of particle design, vessel geometry and fluid dynamics on the efficiency and selectivity of adhesion would provide a foundation upon which to rationally develop this targeting approach. In terms of particle design, parameters would include the polymer, PEG concentration and size. While it was previously shown that adhesion is coupled to particle size for particles in the micrometer range (Shinde Patil et al. 2001), it would be of particular interest to extend such a study to particles in the nanometer range.

As noted above for the PSA-PEG particles, the ligand efficiency was ~16 while the selectivity was ~5. A previous study in the Goetz laboratory used cultured human umbilical vein endothelial cells (HUVECs) and demonstrated that a significantly greater selectivity (~15) is possible for \( \alpha \)-VCAM-1 conjugated biodegradable particles (Sakhalkar et al. 2003). Table 2.1 summarizes ligand efficiencies and selectivities for this and other similar studies. From Table 2.1, it is clear that the targeting efficiency values observed in vivo, in general, tend to lag values observed in vitro e.g. in vivo ligand efficiency of ~9 compared to an in vitro value of ~45 [(Muro et al. 2006a), Table 2.1]; in vivo selectivity of 6 for \( \alpha \)-E-selectin PSA-PEG particles compared to an in vitro value of 12 [(Sakhalkar et al. 2003), Table 2.1]. These results suggest that certain parameters intrinsic to the in vivo environment play a vital role in defining the targeting efficiency. One possible explanation for these differences and the marked difference in selectivity and ligand efficiency observed in the present study is that the wild type
mouse expresses VCAM-1. Indeed, the expression of VCAM-1 on wild type mouse aortic endothelium has been previously documented (Iiyama et al. 1999) and is supported by the data presented in Fig. 2.3 and 2.4 (i.e. compare α-VCAM-1 on wild type mouse aorta to IgG on wild type mouse aorta).

Thus, while it is possible to achieve selective adhesion by targeting VCAM-1, the extent of selectivity may be limited by the differential expression of VCAM-1, i.e. the expression of VCAM-1 on endothelium overlying plaque compared to the expression of VCAM-1 on endothelium overlying healthy tissue. This limitation on selectivity was observed in the femoral artery as shown in Fig. 2.7 where a basal level of VCAM-1 was observed on the femoral artery endothelium.

In drug delivery, an important issue is non-target effects due to non-specific uptake by the reticuloendothelial system (RES). If VCAM-1 is expressed on non-target sites, the biodistribution of the particles may be influenced not only by uptake of the RES but also by non-target expression of VCAM-1. Concerted efforts to characterize the differential expression of surface moieties overlying an inflamed section of a blood vessel wall (e.g. a plaque) versus non-inflamed section of a blood vessel wall (e.g. a non-plaque region) using techniques such as surface glycomics and proteomics of the endothelium will in all likelihood lead to the identification of novel endothelial targets for selective delivery to inflamed tissue (e.g. atherosclerotic tissue).

The work on targeted drug delivery is complementary to targeted imaging (Kaufmann et al. 2007; Kelly et al. 2005; Kelly et al. 2006; Nahrendorf et al. 2006). In
targeted imaging of inflammation, the goal is to exploit the expression of ECAMs to realize concentrations of imaging agents at sites of inflammation, e.g. sites of atherosclerosis. Recently, (Kaufmann et al. 2007) published an elegant study using microbubbles targeted to VCAM-1 for ultrasound imaging of atherosclerosis. Similar to the study in this chapter, they reported that targeted microbubbles exhibit increased accumulation in the aorta of ApoE\(^{-/-}\) mice relative to non-targeted microbubbles, thus supporting the hypothesis that VCAM-1 could be used as a target for particle delivery to aortic plaques with application to drug delivery or imaging. Interestingly, the ligand efficiency and selectivity reported by Kaufmann et al. (2007) were higher than what we report here [\(~45\) and \(~16\) respectively, based on Fig. 2 of (Kaufmann et al. 2007)]. This disparity could be due to differences between the types of particles used (microbubbles vs. biodegradable particles), ligand density on the particles, or the location of the analysis [entire aorta in the present study and thoracic portion of the aorta in (Kaufmann et al. 2007)].

In summary, polymeric particles conjugated with a ligand to VCAM-1 exhibit selective, avid and focal adhesion to sites of atherosclerosis providing strong evidence that VCAM-1 ligand bearing polymeric particles could be used for targeting drugs selectively to atherosclerotic tissue.
CHAPTER 3: DETECTION OF INFLAMED ENDOTHELIAL CELLS IN
WHOLE BLOOD USING QUANTITATIVE REAL-TIME PCR: A POTENTIAL
DIAGNOSTIC/PROGNOSTIC ASSAY FOR ATHEROSCLEROSIS²

Introduction

Mechanisms of atherosclerosis are discussed in detail in Chapters 1 and 2. Certain key processes are reiterated in this chapter as they are vital in understanding the role of inflammation and atherosclerosis in endothelial cell detachment.

As discussed in previous chapters, atherosclerosis is a complex vascular disease that is initiated by the accumulation of lipids at atherosclerosis prone vascular sites (Ross 1999). Lipid accumulation triggers an inflammatory response. This response is characterized by the production of a wide variety of mediators that include pro-inflammatory cytokines (e.g. TNF-α and IL-1β), chemokines (e.g. MCP-1) and reactive oxygen species (Libby 2002; Suvorava and Kojda 2009; Takaya et al. 2007). As a result of the inflammatory response, the vascular endothelium lining the inner walls of the blood vessel expresses high levels of cell adhesion molecules (CAMs). Leukocytes, especially monocytes and T-lymphocytes, from the circulation start interacting with the endothelium through specific CAMs and integrins expressed on their surfaces as a result of the same inflammatory response (Lusis 2000; Ross 1999). This serves as a first step in initiating the leukocyte adhesion cascade leading to the recruitment, firm arrest and

² Prepared for publication as: Detection of Endothelial Cells in Whole Blood Using Quantitative Real-time PCR: A Potential Diagnostic/Prognostic Assay for Atherosclerosis. Sudhir P. Deosarkar, Pooja Bhatt, Christopher J. Lewis and Douglas J. Goetz
migration of leukocytes to the lipid accumulation sites across the endothelium (Eriksson 2003; Libby et al. 2002). This process contributes to the progression of atherosclerosis and the development of atherosclerotic plaques.

Segments of the vascular endothelium in plaque regions are consistently exposed to a variety of pathological insults resulting from pro-inflammatory agents, disturbed blood flow, and alteration in junctional adhesion molecules (Cooke 2003; Ross 1999; Sima et al. 2009; Zhang 2008). These pathological insults cause endothelial dysfunction and damage (Davignon and Ganz 2004; Ross 1999) leading to the shedding of endothelial cells from the blood vessel wall and their entry into the circulating blood (Gao et al. 2008; Woywodt et al. 2002). Additionally, a clinically advanced or vulnerable plaque undergoing surface erosion can also potentially contribute to the shedding of endothelial cells into the circulating blood (Libby 2002; Virmani et al. 2000). Both mechanisms of endothelial cell detachment, namely pathological insult and surface erosion, separately or together, may contribute to a substantial increase in the number of endothelial cells circulating in an atherosclerotic patient’s blood. Thus, a reliable and effective assay that detects, characterizes and quantifies circulating endothelial cells (CECs) might serve as a valuable diagnostic/prognostic for atherosclerosis.

Various assays have been developed in the past to detect and enumerate CECs in a variety of vascular disorders. The two most widely used assays for CEC detection are multichannel flow cytometry and immuno-magnetic bead separation (Clarke et al. 2008; Duda et al. 2007; Goon et al. 2006; Makin et al. 2004; Mariucci et al. 2009; Widemann et al. 2008). Although these assays were shown to be successful in studies involving the
detection of endothelial cells (Mancuso et al. 2001; Widemann et al. 2008), a recent study indicated discrepancies when these two assays were compared for the quantification of circulating endothelial cells (Clarke et al. 2008). Additionally, some studies indicated that a flow cytometry based assay could lead to false positives (Goon et al. 2006; Khan et al. 2005; Strijbos et al. 2007). Both assays, namely flow cytometry and immuno-magnetic bead separation, are based on the detection of proteins expressed on the endothelial cell surface using a suitable ligand. The discrepancies associated with these assays and the fact that they utilize protein expression prompted us to develop a potentially more sensitive, mRNA based real-time PCR (polymerase chain reaction) assay for the detection of endothelial cells in whole blood.

Successful development of a real time PCR based assay for the detection of endothelial cells from whole blood requires selection of appropriate endothelial cell specific markers. Also, in order to predict the extent of vascular damage in atherosclerosis, the assay should be able to address two aspects of vascular damage as they relate to atherosclerosis viz., the number of circulating endothelial cells and the phenotype of the detected circulating endothelial cells. For the determination of the number of circulating endothelial cells, an endothelial marker would be required that is endothelium specific in the physiological environment such as blood. Several endothelial markers have been identified and studied extensively. These include PECAM-1, CD34, CD105, MCAM, claudin-5, and VE-cadherin (Breviario et al. 1995; Middleton et al. 2005; Vestweber 2008). Not all of these markers could serve as endothelial cell specific markers in a given physiological setting such as blood. For example, PECAM-1 may not
be a suitable endothelial marker for the detection of endothelial cells from whole blood since it is also expressed by subsets of leukocytes and platelets (Woodfin et al. 2007). In contrast, MCAM (also known as CD146, Mel-CAM, MUC-18, S-Endo-1 and A32 antigen), a ~113 kDa membrane glycoprotein originally discovered as melanoma associated antigen (Lehmann et al. 1987; Sers et al. 1994; Shih 1999), has been extensively studied as an endothelial marker (Bardin et al. 2001; Bardin et al. 2003; Widemann et al. 2008). Additionally, various studies have successfully used MCAM as a marker for the detection of circulating endothelial cells from whole blood in a variety of diseases that lead to vascular damage. Examples of such studies include the detection of CECs in non-ST-elevation acute coronary syndrome patients (Quilici et al. 2004), breast cancer patients (Furstenberger et al. 2005), and chronic lymphocytic leukemia patients (Go et al. 2008). These studies, along with the fact that MCAM is specific to endothelial cells in peripheral blood, indicate that MCAM could serve as an ideal marker for determining the number of endothelial cells.

Another important aspect of the real-time PCR assay in the present study is the determination of the phenotype of the detected endothelial cells. This dictates selection of an endothelial marker that is associated with the development and progression of atherosclerosis. Vascular cell adhesion molecule-1 (VCAM-1) has been extensively studied with regard to atherosclerosis development and progression since it was first discovered as an athero-ELAM (Cybulsky and Gimbrone 1991). Indeed, VCAM-1 has been shown to be highly expressed at sites of atherosclerosis (Deosarkar et al. 2008; Iiyama et al. 1999; Nakashima et al. 1998) and its expression by the endothelial cells
within the plaque regions has been suggested as an important characteristic of the atherosclerotic phenotype (O’Brien et al. 1993). This increased expression of VCAM-1 within the plaque region is mainly due to the pro-inflammatory cytokines produced during atherogenesis (Branen et al. 2004; Kirii et al. 2003). Thus, the endothelial cells originating from an atherosclerotic plaque may exhibit a significantly higher expression of VCAM-1 than the endothelial cells originating from a healthy vascular site. This suggests that by assessing the VCAM-1 expression of CECs (i.e. inflamed or non-inflamed state) in an atherosclerotic patient’s blood using a real-time PCR assay, it may be possible to gain insight into the extent of damage in atherosclerosis and determine the associated clinical risk.

The goal of the present study is to develop a dual marker real-time PCR assay capable of detecting, enumerating and characterizing endothelial cells in whole blood. We explored the use of MCAM and VCAM-1 as markers for endothelial cells and the inflamed state of the endothelial cells respectively. Our studies indicate that a real-time PCR assay developed here may serve as a promising diagnostic tool for detecting VCAM-1 positive CECs in atherosclerotic patients.
Materials and Methods

Materials

Primers for the human genes VCAM-1 (assay id: Hs00365485_m1), MCAM (assay id: Hs 00174838_m1), ACTB (beta actin) and RPLPO were from Applied Biosystems (Foster City, CA). High capacity cDNA synthesis kit and TaqMan gene expression master mix were from Applied Biosystems (Foster City, CA). RNeasy® plus mini kit, Qiashredder™ columns and PAXgene™ blood RNA kit were purchased from Qiagen (Valencia, CA). rhTNF-α, rhIL-1β, rhMCP-1, were from EMD Chemicals, Inc. (Gibbstown, NJ). LPS (E.coli, O111:B4), ethanol, gelatin, β-mercaptoethanol, heparin were from Sigma-Aldrich (St. Louis, MO). rhIL-6 and rhIFN-γ were from R & D systems (Minneapolis, MN). Endothelial cell culture reagents were as described previously (Dagia and Goetz 2003). PAXgene™ blood RNA tubes were from VWR (West Chester, PA).

Cell culture

HUVEC media was prepared as previously described (Dagia and Goetz 2003). For all experiments, HUVEC were used at passage three through five. For gene expression studies with pure HUVEC, HUVEC were cultured in 6-well plates, whereas for blood spiking studies, HUVEC were cultured in T-25 flasks. 6-well plates and T-25 flasks were pretreated with 0.1% gelatin.
RNA purification

For studies with pure HUVEC, HUVEC were treated for 4hrs with IL-1β, TNF-α, INF-γ, IL-4, IL-6, MCP-1, or LPS. After treatment, the cells were washed twice with PBS. Total RNA was isolated from HUVEC using RNeasy® plus mini kit according to the manufacture’s protocol.

For blood spiking studies, an IRB approved protocol was used to draw whole blood from healthy volunteers into PAXgene™ blood RNA tubes. HUVEC were treated with TNF-α (25ng/ml), IL-1β (50U/ml) or LPS (50ng/ml) for 4 hours, trypsinized, washed and resuspended in RNase-free water. A predetermined number of HUVEC (treated or untreated) were spiked into whole blood. As a negative control, some blood tubes received equal volumes of RNase-free water. Total RNA was isolated using PAXgene™ blood RNA kit according to the manufacturer’s protocol.

RNA was quantified using a spectrophotometer. The ratio of absorbance at 260 nm to the absorbance at 280 nm was used to asses the purity of the RNA samples. Samples yielding a ratio of 1.8-2.0 were considered pure. Purified RNA was stored at -80 °C until used for further assays/experiments.

cDNA synthesis and real-time PCR analysis

First-strand cDNA for PCR template was synthesized from 500-1500 ng of total RNA using high capacity cDNA synthesis kit according to the manufacturer’s protocol. Stratagene MaxPro 3000p real-time PCR system was used for the PCR. Thermal settings for the PCR reaction were: one cycle of AmpliTaq Gold® enzyme activation at 95°C for
10 min followed by 40 cycles of 15 second denaturation at 95°C and 1 minute annealing/extension at 60 °C. VCAM-1 primers were chosen such that they were able to anneal and amplify both VCAM-1 mRNA transcript variants (Cybulsky et al. 1991). PCR efficiency for all primers was checked by generating standard curves using serial dilutions of cDNA. Since the efficiency was similar and close to 100% (data not shown), for all cases, 100% efficiency was considered in calculating fold expression of VCAM-1 and MCAM mRNA. To calculate relative fold expression of VCAM-1 and MCAM mRNA, \(2^{-\Delta \Delta C_t}\) method was used for pure HUVEC while \(2^{-\Delta C_t}\) method was used for whole blood spiked with HUVEC where \(\Delta C_t\) and \(\Delta \Delta C_t\) are calculated using following equations (Livak and Schmittgen 2001).

\[
\Delta C_t = C_{t\text{arget gene}} - C_{t\text{housekeeping gene}} \\
\Delta \Delta C_t = \Delta C_{t\text{reatment}} - \Delta C_{t\text{control}}
\]

A flow chart representing experimental steps followed in this study is shown in Fig. 3.1.

**Statistics and data analysis**

A student’s t-test was used for comparing two means. For experiments involving two factors, a two-factor ANOVA along with a Tukey-Kramer multiple comparison test were used. ANOVA and Tukey-Kramer tests were performed using NCSS 2004 statistical analysis software (Kaysville, UT). \(p<0.01\) was considered significant.
Figure 3.1: Experimental steps in the determination of VCAM-1 and MCAM mRNA expression.

1. HUVEC culture and treatment with cytokines/mediators
2. HUVEC spiked in blood or used alone
3. RNA extraction using Qiagen™ or Paxgene™ RNA kit
4. cDNA synthesis using high capacity cDNA synthesis kit
5. Calculate fold expression using \( \Delta \Delta Ct \) or \( \Delta Ct \) method
6. Quantitative real time PCR with fixed amount of cDNA in duplex reactions; primers for MCAM, VCAM-1, \( \beta \)-actin, and RPLPO used
Results

The basal expression of MCAM in HUVEC is significantly higher than VCAM-1

HUVEC are commercially available, highly robust and easy to work with. In addition, Goetz laboratory and others have characterized adhesion molecule (e.g. VCAM-1) expression at the protein level (Dagia and Goetz 2003; Sakhalkar et al. 2003). For these reasons, HUVEC were chosen as a model endothelial cell for the studies in this chapter. First, the basal expression of MCAM and VCAM-1 mRNA in HUVEC was investigated. cDNA was synthesized from total RNA isolated from untreated HUVEC. Real-time PCR was performed with the generated cDNA using primers for MCAM and VCAM-1. mRNA expression for both MCAM and VCAM-1 were calculated relative to β-actin (a housekeeping gene) using $2^{-\Delta\Delta C_t}$ method. As shown in Fig. 3.2, in the absence of stimulation with cytokines or mediators, MCAM mRNA is abundant and constitutively expressed in HUVEC at a significantly higher level compared to VCAM-1.

Cytokine treatment of HUVEC has little, if any effect on MCAM but causes significant induction of VCAM-1

After determining the basal expression levels of MCAM and VCAM-1 mRNA in HUVEC, next their response to cytokine treatment was investigated. TNF-α and IL-1β have been implicated in atherosclerosis in inducing adhesion molecules such as VCAM-1 and LPS has been shown to increase lesion size in atherosclerosis (Branen et al. 2004; Kirii et al. 2003; Marui et al. 1993). Additionally, they are well studied for their effect on
VCAM-1 expression by HUVEC. But the effect of these mediators on MCAM mRNA expression by HUVEC is not well studied. Hence, the effect of TNF-α, IL-1β and LPS on MCAM expression by HUVEC was investigated. As shown in Fig. 3.3A, 3.3B and 3.3C VCAM-1 expression increased after treatment of HUVEC with all the three mediators in line with previous studies (Dagia and Goetz 2003; Marui et al. 1993). mRNA expression of VCAM-1 relative to β-actin was 2571±758, 267±179, and 7±3 for HUVEC treated with TNF-α (25ng/ml), IL-1β (50U/ml), and LPS (50ng/ml) respectively. Note that the VCAM-1 mRNA expression was not a function of the mediator concentration for the range of concentrations studied. On the other hand, TNF-α, IL-1β and LPS had no significant effect on MCAM expression by HUVEC (Fig. 3.3A, 3.3B and 3.3C).

Figure 3.2: MCAM has a higher basal expression than VCAM-1 in HUVEC. mRNA expression is relative to β-actin. Error bars indicate SEM (n=3). * Significantly different from other bar (P<0.01).
Figure 3.3: VCAM-1 is highly responsive to cytokine treatment whereas MCAM is not.

VCAM-1 and MCAM mRNA expression in (A) TNF-α, (B) IL-1β, (C) LPS (strain: E Coli 0111:B4) and (D) IL-6, IL-4, MCP-1, IFN-γ treated HUVEC. HUVEC treatment was for 4hr at 37°C. mRNA expression is relative to β-actin and untreated HUVEC. Error bars indicate SEM (n=3 experiments). VCAM-1 mRNA expression for TNF-α, IL-1β, LPS treatments (black bars) was significantly (P<0.01) higher than MCAM mRNA expression (white bars).
Since the present study serves as a first step towards developing a blood based real-time PCR diagnostic assay, it was important to study the effect of additional physiologically relevant cytokines that could be present in clinical blood samples and may potentially affect the expression of VCAM-1 and MCAM. Hence, in addition to TNF-α, IL-1β and LPS, the effect of IL-6, IL-4, MCP-1 and IFN-γ on MCAM and VCAM-1 mRNA expression was also investigated. The results of this study, as shown in Fig. 3D, indicate that none of the cytokines studied had a significant effect on MCAM mRNA expression by HUVEC. Also, as can be seen from Fig. 3.3D, VCAM-1 expression did not change significantly for IL-6, IL-4, MCP-1 and IFN-γ treatments.

**VCAM-1 expression in HUVEC decreases with longer incubation times with cytokines while MCAM expression stays the same**

As the results in Fig. 3.3 indicated that both VCAM-1 and MCAM mRNA expression in HUVEC was dosage independent, it was important to investigate if the incubation time with cytokines had any effect on these expressions. In a given physiological environment, endothelial cells within a plaque region and endothelial cells in the circulation may be exposed to cytokines for a variety of time durations. This could serve as an important factor in determining the inflamed state of endothelial cells. Hence, next the effect of TNF-α, IL-1β and LPS on the expression of VCAM-1 and MCAM mRNA after 4, 12 and 24 hour incubation was studied. The concentrations used in these experiments were 50ng/ml for TNF-α and LPS and 50U/ml for IL-1β. As shown in Fig. 3.4A, expression of VCAM-1 mRNA is highest at 4 hour incubation and decreases with
higher incubation times. For example, VCAM-1 mRNA expression was 2669±728 and 88±19 in case of HUVEC treated with 50ng/ml TNF-α for 4 and 24 hours respectively. Also, as can be seen in Fig. 3.4A, HUVEC exhibited a cytokine dependent expression of VCAM-1 similar to the trend observed in Fig. 3.3. On the other hand, MCAM mRNA had no significant change in its expression in HUVEC for any of the treatment conditions (Fig. 3.4B).
Figure 3.4: Incubation time for cytokine treated HUVEC affects VCAM-1 expression but not MCAM expression.

Effect of incubation time on (A) VCAM-1 and (B) MCAM mRNA expression in TNF-α, IL-1β, and LPS (strain: E Coli 0111:B4) treated HUVEC (4, 12 and 24 hr at 37°C), (C) MCAM mRNA expression in IL-6, TNF-α, IL-4, IL1-β, MCP-1 treated HUVEC (24 hr and 48 hr at 37°C). mRNA expression is relative to β-actin and untreated HUVEC. Error bars indicate SEM (n=3 experiments for A and B and n=3 PCR reactions for C). *4 hour treatment is significantly different from 12 and 24 hour treatments (P<0.01).
Real-time PCR of MCAM and VCAM-1 in whole blood enables detection of endothelial cells and determination of their inflamed state

Results shown in Figs. 3.2 through 3.4 established that physiologically relevant inflammatory cytokines do not have a significant effect on MCAM mRNA expression in HUVEC. Thus, MCAM may serve as an effective endothelial marker in a whole blood real-time PCR based assay for predicting vascular damage in atherosclerosis.

To further test the effectiveness of MCAM and VCAM-1 as markers of vascular damage, blood-based studies were devised. Cytokine treated and untreated HUVEC were spiked into whole blood collected from healthy donors. Separate sets of HUVEC were treated with TNF-α, IL-1β and LPS and then spiked into whole blood. The results of these studies are shown in Fig. 3.5. Fig. 3.5A, 3.5B and 3.5C show relative mRNA expression plotted against the number of HUVEC spiked. Such data can serve as standard curves for determining the number of HUVEC from an unknown blood sample. A linear regression model was used, as shown in Equation 3.3, to fit the standard curves of MCAM and VCAM-1 mRNA expression in HUVEC spiked blood. The values for the slope and the correlation coefficient for each condition are listed in Table 3.1. For all three treatments (TNF-α, IL-1β and LPS), MCAM mRNA expression in treated and untreated HUVEC and VCAM-1 mRNA expression in treated HUVEC correlated with the number of HUVEC spiked (Fig. 3.5A, 3.5B and 3.5C), the range of correlation coefficients (Table 3.1) was 0.87-0.99 and 0.83-0.95 for MCAM and VCAM-1 respectively. In contrast, VCAM-1 mRNA expression in untreated HUVEC did not correlate with the number of HUVEC spiked (Fig. 3.5A, 3.5B and 3.5C) as indicated by
the correlation coefficients in Table 3.1. Also, the slope of the standard curve for VCAM-1 was highest in the case of TNF-α treatment compared to IL-1β and LPS treatments indicating a cytokine dependent VCAM-1 mRNA expression. In contrast, the slope of the standard curve for MCAM stayed relatively constant for all treatments indicating a cytokine independent MCAM expression.

\[ mRNA\ Expression = (Slope) \times (HUVECs/\mu l) \]  

(3.3)

To assess the VCAM-1 expression by endothelial cells, a parameter termed the endothelial VCAM-1 to MCAM ratio (EVMR) was defined, as given in Equation 3.4 below. The results are shown in Fig. 3.6A, 3.6B and 3.6C. In the case of blood spiking studies with TNF-α and IL1-β treated HUVEC, the EVMR for blood spiked with treated HUVEC was significantly higher than that for blood spiked with untreated HUVEC (Fig. 3.6A and 3.6B), whereas in the case of blood spiking studies with LPS pretreated HUVEC, there was no significant difference between EVMR for blood spiked with treated and untreated HUVEC (Fig. 3.6C). It is also important to note that EVMR in blood spiked with TNF-α treated HUVEC approaches 1.0 (Fig. 3.6A). This indicates that the maximal VCAM-1 expression approaches the basal MCAM expression in HUVEC.

\[ EVMR = \frac{VCAM-1\ mRNA\ Expression}{MCAM\ mRNA\ Expression} \]  

(3.4)
Figure 3.5: Standard curves for relative expression of VCAM-1 and MCAM in HUVEC spiked blood.

(A) Blood was spiked with TNF-α (50ng/ml) treated and untreated HUVEC (1-16 HUVEC/μl). (B) Blood was spiked with IL-1β (50U/ml) treated and untreated HUVEC (1-16 HUVEC/μl). (C) Blood was spiked with LPS (50ng/ml) treated and untreated HUVEC (1-16 HUVEC/μl). Error bars indicate SEM (n=3 PCR reactions). For all treatments, VCAM-1 mRNA expression was significantly higher (P<0.01) in blood spiked with treated HUVEC compared to blood spiked with untreated HUVEC. THB: Treated HUVEC in Blood and UHB: Untreated HUVEC in Blood.
Figure 3.6: Endothelial VCAM-1 to MCAM Ratio (EVMR) plots for whole blood spiked with HUVEC.

(A) Blood was spiked with TNF-α (50ng/ml) treated and untreated HUVEC (1-16 HUVEC/μl). (B) Blood was spiked with IL-1β (50U/ml) treated and untreated HUVEC (1-16 HUVEC/μl). (C) Blood was spiked with LPS (50ng/ml) treated and untreated HUVEC (1-16 HUVEC/μl). Error bars indicate SEM (n=3 PCR reactions). For all treatments, EVMR values were significantly higher (P<0.01) in blood spiked with treated HUVEC compared to blood spiked with untreated HUVEC. THB: Treated HUVEC in Blood and UHB: Untreated HUVEC in Blood.
Table 3.1

Slopes and correlation coefficients for standard curves in blood spiking studies

Equation 3.3 was used to determine the slope of the standard curves in Figure 3.5. Correlation coefficient <=0 indicates no correlation between number of HUVEC and the mRNA (VCAM-1 or MCAM) expression.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>HUVEC Treatment</th>
<th>VCAM-1</th>
<th>MCAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Correlation Coefficient</td>
<td>Slope</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Treated</td>
<td>0.0243</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Unreated</td>
<td>$1\times10^{-5}$</td>
<td>-1.25</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Treated</td>
<td>0.0019</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Unreated</td>
<td>$1\times10^{-5}$</td>
<td>-5.42</td>
</tr>
<tr>
<td>LPS</td>
<td>Treated</td>
<td>0.0003</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>Unreated</td>
<td>$3\times10^{-5}$</td>
<td>-1.23</td>
</tr>
</tbody>
</table>
To assess the extent of activation of endothelial cells, an additional parameter called Endothelial Cell Activation Index (ECAI) was defined as given in Equation 3.5 below. For the present studies, ECAI ranged between 1224±301 and 1940±605. Fig. 3.7 shows ECAI for pure HUVEC treated with TNF-α, IL-1β and LPS as well as for whole blood spiked with TNF-α, IL-1β and LPS treated HUVEC. For each treatment, ECAI for pure HUVEC and for blood spiked with HUVEC are not significantly different. This indicates that the real-time PCR system developed here is able to determine the activation state of endothelial cells in whole blood where the ECAI values are comparable for the samples containing pure HUVEC and HUVEC spiked whole blood.

\[
ECAI = \frac{EVMR_{\text{in treated HUVEC or blood}}}{EVMR_{\text{in untreated HUVEC or blood}}} \tag{3.5}
\]
Figure 3.7: Comparison of Endothelial Cell Activation Index (ECAI) for HUVEC and HUVEC spiked blood.

ECAI for blood spiked with HUVEC and pure HUVEC. Error bars indicate SEM (n=15 for blood and n=3 for pure HUVEC). $Significantly different from IL-1β and LPS treatments ($P<0.01$). *Not significantly different from the white bar for the same treatment ($P>0.01$).
Discussion

In a variety of vascular diseases, such as atherosclerosis, a patho-physiological insult has been implicated as a possible cause for the detachment of endothelial cells from a vascular site of inflammation (Gao et al. 2008; Ross 1999; Woywodt et al. 2002). An assay that can characterize the detached endothelial cells could be useful in predicting vascular damage in atherosclerosis and may serve as a diagnostic tool in a clinical setting. Hence, in the present study, a real-time PCR based assay was developed to detect inflamed endothelial cells in whole blood.

As discussed in the beginning of this chapter, determination of the number and activation state of the circulating endothelial cells (CECs) could serve as a marker for the extent and severity of the vascular disease. In the past, various studies attempted to determine the correlation between the number of CECs and vascular disease. A number of studies associated increased levels of CECs with a variety of diseases that include vascular disorders as well as diseases leading to vascular disorders; examples of such diseases include vasculitis (Clarke et al. 2008), atherosclerosis (Makin et al. 2004), breast cancer (Furstenberger et al. 2005; Mancuso et al. 2001), lymphoma (Mancuso et al. 2001), acute coronary syndrome (Boos et al. 2008; Quilici et al. 2004) and rheumatoid arthritis (Foster et al. 2009). These studies showed that there is indeed a correlation between the number of CECs in blood samples from patients with vascular disease; higher number of CECs meant extensive vascular damage or vascular remodeling.

In all of the studies, MCAM has been the marker of choice for detecting endothelial cells and as discussed in the beginning of this chapter, various methods with
MCAM as a marker have been developed to assess CEC number. Recently, real-time PCR has been suggested as a viable technique for detecting and enumerating CECs because of its greater efficiency and accuracy. One recent study used real-time PCR to enumerate CECs in breast cancer patients and also compared it with four-color flow cytometry results (Furstenberger et al. 2005). The use of MCAM as an endothelial marker is justified for two reasons, one that it is specific to endothelial cells in a vascular environment and two that it has a high basal expression in endothelial cells. In the present study, the basal expression of MCAM with VCAM-1 in untreated endothelial cells was compared. As shown in Fig. 3.2, we observed that MCAM indeed has a higher basal expression than VCAM-1. This suggests that, in addition to being an endothelial marker, in real-time PCR, MCAM could yield a higher sensitivity for the endothelial cell detection due to its higher basal expression.

MCAM has been widely used as a CEC marker in several different techniques including flow cytometry, magnetic bead separation and real-time PCR. But its expression has not been well studied with respect to the complex vascular environment. In an inflammatory setting such as atherosclerosis, the inflamed vascular site could present an environment that is rich in pro-inflammatory cytokines which may play a role in altering endothelial cell biology. For example, pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, IFN-γ, MCP-1 have been shown to play a critical role in the development and progression of atherosclerosis (Hansson 2001; Tedgui and Mallat 2006; Young et al. 2002) through changes in the expression of adhesion molecules such as VCAM-1 (Cybulsky and Gimbrone 1991; Deosarkar et al. 2008; Iiyama et al. 1999;
Nakashima et al. 1998). Such a cytokine rich environment may also have an effect on MCAM expression. Given the previous observations that MCAM expression is elevated in certain inflammatory settings such as inflammatory bowel disease (Bardin et al. 2006) and inflammatory skin disease (Weninger et al. 2000), downregulated in breast carcinomas (Shih et al. 1997) and has a role in homophilic and heterophilic interactions in cell-cell adhesion (Guezguez et al. 2007), in the current study, it was postulated that the expression of MCAM could be regulated by inflammatory cytokines.

Previously, a study investigated the effect of stimulants such as TNF-α, IL-1β, LPS and IL-6 on MCAM expression by HUVEC using flow cytometry (Bardin et al. 1996). Although, this study showed that these stimulants had little or no effect on MCAM protein expression by HUVEC, it was necessary to test their effect on mRNA level since protein expression can be regulated post-transcriptionally. Hence, the effect of cytokines on endothelial MCAM mRNA expression was investigated. For this purpose, a panel of cytokines was used that have been implicated in atherosclerosis. Our results, shown in Figures 3.3 and 3.4, indicated that none of the cytokines had a significant effect on MCAM expression by HUVEC. This suggests that in a given inflammatory setting such as atherosclerosis, the endothelial expression of MCAM would not change significantly. Hence, MCAM may serve as an effective marker for endothelial cells particularly in the inflamed vascular environment.

In addition to the detection of endothelial cells in whole blood by assessing MCAM expression, the endothelial cell phenotype was further characterized by assessing its VCAM-1 expression and how it would correlate with the CEC number. As discussed
previously, VCAM-1 plays a major role in the development and progression of atherosclerosis. Hence, in this study, it was hypothesized that the endothelial cells detached from an inflamed vascular site in atherosclerosis would exhibit a distinct VCAM-1 expression profile, generally higher than normal. TNF-α, IL-1β and LPS treated and untreated HUVEC were spiked in whole blood and studied the expression of both MCAM and VCAM-1 mRNA using real-time PCR. As shown in Fig. 3.5A, 3.5B and 3.5C, MCAM mRNA expression correlated with the number of spiked HUVEC. This, or a similar presentation for other endothelial cell types, could serve as a standard curve for determining the number of endothelial cells in whole blood from MCAM mRNA expression. Additionally, VCAM-1 mRNA expression in blood spiked with cytokine treated HUVEC was many orders of magnitude higher than the blood spiked with untreated HUVEC (e.g. Fig. 3.5A and 3.5B).

Thus, in addition to determining the number of endothelial cells in whole blood by comparing MCAM mRNA expression in a test sample with that in the standard curve, VCAM-1 expression would suggest the activation state of the detected endothelial cells from the VCAM-1 standard curves as well as from EVMR. Also the mRNA expression and EVMR values of a test sample could be used to predict ECAI which will indicate the extent of activation of detected endothelial cells in a test blood sample.

In conclusion, a quantitative real-time PCR based assay was developed utilizing MCAM and VCAM-1 as the markers for endothelial cell and its activation state respectively. In the study presented in this chapter, it was established that MCAM could be used as an endothelial marker in a vascular environment and that VCAM-1 could be
effectively used to determine the activation state of detected endothelial cells. Thus, the real-time PCR assay developed here is a first step towards a diagnostic tool for assessing the state of vascular damage in atherosclerosis.
CHAPTER 4: MATHEMATICAL ANALYSIS OF THE ADHESION OF LIGAND CONJUGATED PARTICLES TO AN ATHEROSCLEROTIC PLAQUE

Introduction

In previous chapters, the mechanisms of atherosclerosis were discussed in the context of the formation of lipid laden plaques. A polymeric particle based delivery system for targeting atherosclerotic sites is presented in Chapter 2 where the results indicated that VCAM-1 conjugated polymeric particles exhibit enhanced adhesion to sites of atherosclerosis. This and other particle based targeting studies indicate that the present particle based targeting systems need further improvements in areas such as particle design and surface chemistries to achieve better targeting efficiencies (Dickerson et al. 2001; Sakhalkar et al. 2003; Sakhalkar et al. 2005; Shinde Patil et al. 2001; Soppimath et al. 2001; Spragg et al. 1997; Stahn et al. 2001). In addition to improving particle design and surface chemistry, elaborate studies would be required to understand the \textit{in vivo} environment at the targeting sites within the vasculature.

As noted in Chapter 2, the results were comparable to previous \textit{in vivo} particle adhesion studies (Sakhalkar et al. 2003; Sakhalkar et al. 2005) in the Goetz laboratory. This was surprising given the differences in the vascular beds studied. Although these studies indicated similar effectiveness in overall performance of the particle based targeting system, a detailed analysis of particle adhesion within a target site would help in delineating the existing nature of local adhesion profiles and possibly help achieve better targeting efficiencies.
The analysis of local adhesion profiles can conceivably be achieved by experimental or mathematical approaches. A plethora of experiments may be designed and performed to assess the effects of independent governing parameters and processes that affect the adhesion profiles within the target site. But often times, such an approach can prove cumbersome and expensive. On the other hand, a mathematical analysis of particle based targeting to the site of inflammation could prove very insightful in understanding the effects of parameters that govern the targeting efficiency.

Various approaches have been used in the past to develop mathematical models to understand the effect of underlying processes and process parameters on particle adhesion efficiencies. The Hammer group developed a model called adhesive dynamics to study the effect of forces due to hydrodynamic and biomolecular processes on receptor mediated adhesion (Caputo and Hammer 2005; English and Hammer 2004; Hammer and Apte 1992; King and Hammer 2001). In a recent study from the same group, the researchers developed a more general model to study nanoparticle adhesion involving biomolecular interactions and validated the model using data from an \textit{in vitro} system (Haun and Hammer 2008). Another research group developed mathematical models to understand the hydrodynamic effects on intercellular bond formation in cell adhesion (Neelamegham et al. 1997; Shankaran and Neelamegham 2004; Zhang and Neelamegham 2002). These studies were able to gain insightful information from devising mathematical models when studying particle adhesion to receptor bearing surfaces. Thus, a mathematical analysis alone or in combination with experiments has always proved beneficial in understanding the processes existent within the system.
Development of a mathematical model to analyze a given system depends on a variety of factors and as discussed in the previous paragraphs, this development could be approached in a variety of ways. The goal of the present analysis was to study which parameters are critical to the system under consideration and how they affect targeting efficiencies. Efficient delivery of polymeric particles to vascular sites of inflammation is governed by a variety of factors. These include reaction rates between the ligands on the particles and the receptors on the endothelium, transport of particles to the vessel wall by convection and diffusion, shape of the vessel lumen, fluid characteristics and particle size (Haun and Hammer 2008; Horner et al. 1995; Lee and Xu 2002; Shinde Patil et al. 2001; Tees et al. 2002; Zhang and Neelamegham 2002). When determining targeting efficiency in the case of atherosclerosis, a major factor that could influence the particle adhesion to the inflamed vessel wall is the constriction of the vessel lumen due to the presence of an atherosclerotic plaque. An atherosclerotic plaque will alter the flow profile in the vessel and hence could affect the adhesion of particles (Zarins et al. 1983). Indeed, the results of various previous studies, which developed mathematical models for studying hydrodynamics in stenosed vessels, indicated that the presence of a stenosis due to plaque formation significantly changes flow profiles in the stenosed region of the vessel (Berger and Jou 2000; Lee and Xu 2002; Lee 2002; Libby et al. 1997; Padmanabhan 1980).

Hence, in order to understand the effect of the presence of a plaque and other governing parameters on the adhesion of ligand conjugated particles, a mathematical model was devised and solved numerically.
Mathematical Model

A previously published study by Horner et al. (1995) used basic mass continuity, Navier-Stokes and species continuity equations to develop velocity and concentration profiles for their system. They studied the deposition of low density lipoprotein (LDL) to the arterial wall in the presence of a stenosis. A similar mathematical analysis was performed in the present study as outlined below to study the adhesion of ligand conjugated particles to an atherosclerotic plaque. For this purpose, mass continuity, Navier-Stokes and species continuity equations are adapted from Horner et al. (1995) and modifications are highlighted wherever our approach differed from Horner et al. (1995).

Vessel Geometry

Horner et al. (1995) used a discontinuous function to define the stenosis geometry. In the study below, a plaque bearing vessel is modeled as a cylindrical tube with a bell-shaped constriction. The constriction is assumed to be symmetric with the vessel axis for computational simplicity. Hence, a 2-dimensional approach can be considered in developing the model equations. The model geometry of the plaque bearing vessel is shown in Fig. 4.1. The plaque geometry is defined by Equation 1 given in Padmanabhan (1980) which gives the radius of lumen (or the shape of vessel lumen) as a function of position along the lumen length and assumes a bell shaped stenosis representing a plaque.

\[
R_z = R_0 - H \left(1 + \cos \left(\frac{\pi z}{2X_0}\right)\right), \text{ for } -X_0 \leq z \leq X_0, \text{ from Padmanabhan (1980)} \tag{4.1}
\]
$2H$ and $2X_0$ are respectively the height of stenosis at the center of the plaque and the spread of the plaque inside the vessel, as shown in the Fig. 4.1.

**Fluid dynamics**

Using the Horner et al. (1995) approach but with a different stenosis model geometry (shown in Fig. 4.1 and defined by Equation 4.1), velocity profiles can be developed. The equations that define the flow fields are the equation for conservation of mass, Equation 4.2 (equation 2 in Horner et al. (1995)), and the Navier-Stokes equation of motion, Equation 4.3 (equation 3 in Horner et al. (1995)). These equations can also be found in Transport Phenomenon (Bird et al. 2002) and (Jen-Shih Lee 1970).

\[
\frac{1}{r} \frac{\partial (rv_r)}{\partial r} + \frac{\partial v_z}{\partial z} = 0 \quad (4.2)
\]

\[
\frac{\partial v}{\partial t} + (v \cdot \nabla)v = \frac{1}{\rho}(-\nabla p + \nabla \cdot \tau) \quad (4.3)
\]

Where, $v$ is the velocity vector, $p$ is the pressure, $\rho$ is the density of the fluid and $\tau$ is the shear stress tensor.

A simplified version of the $z$-component of the Navier-Stokes equation can be written as follows (Bird et al. 2002; Horner et al. 1995).

\[
\frac{\mu}{r} \frac{\partial}{\partial r} \left( r \frac{\partial v_z}{\partial r} \right) = -\frac{dP}{dz}, \text{ from Horner et al. (1995)} \quad (4.4)
\]

Where, \( \frac{dP}{dz} = \frac{8\mu Q}{\pi R_z} \), from Horner et al. (1995) \quad (4.5)
Figure 4.1: Schematic of plaque bearing vessel. An atherosclerotic plaque is modeled as a bell shaped constriction.

The radius of the constriction is defined by Equation 4.1.
Equation 4.4 is solved using the following boundary conditions and Equation 4.5 to obtain the axial velocity profile.

B.C. 1: \( v_z = 0 \) at \( r = R_z \), and

B.C. 2: \( v_z = \text{function of } z \), at \( r = 0 \)

B.C. 1 in the present analysis differs from Horner et al. (1995) and hence also changes the function for B.C. 2.

Thus, the axial velocity profile given below has a similar form as that given in Horner et al. (1995) but with a different stenosis model given by Equation 4.1.

\[
v_z = \frac{2Q}{\pi R_z^2} \left[ 1 - \left( \frac{r}{R_z} \right)^2 \right]
\]

Where, \( R_z \) is given by Equation (4.1)

From this point onwards our model starts to substantially diverge from Horner et al. (1995). As the first difference, in the following analysis, a maximum axial velocity (Equation 4.7) was used to define the characteristic axial velocity that would be used in the numerical analysis in the following sections. For the purpose of the present analysis, the maximum velocity is defined at \( r = 0 \) and \( z = 0 \), as given below

\[
v_{z\text{max}} = \frac{2Q}{\pi (R_0 - 2H_0)^2}
\]

Estimation of the radial velocity profiles is important for the present analysis due to the presence of the stenosis in the flow path. The radial velocity is estimated in a similar fashion as Horner et al. (1995) by starting with the mass continuity equation, Equation 4.2 with the incorporation of the axial velocity profile given in Equation 4.6.
Thus, the radial velocity profile for the present analysis becomes,

$$v_r = \frac{Q}{\pi R_z^2} \left[ \left( \frac{r}{R_z} \right)^3 - \left( \frac{r}{R_z} \right)^3 \right] dR_z dz$$  \hspace{1cm} (4.8)

$$\frac{dR_z}{dz}$$ is estimated from Equation 4.1 and substituted in Equation 4.8 to give,

$$v_r = \left[ \left( \frac{r}{R_z} \right)^3 - \left( \frac{r}{R_z} \right)^3 \right] \left[ \frac{QH_o}{2R_z^2 X_0} \sin \left( \frac{\pi z}{2X_0} \right) \right]$$  \hspace{1cm} (4.9)

Thus, the axial and radial velocity profiles given above have a slightly different form from those given in Horner et al. (1995).

**Mass Transport in the Plaque Region**

Horner et al. (1995) used the standard species continuity equation for developing concentration profiles. A similar approach was followed for developing particle concentration profiles in the present mathematical analysis. Thus the species continuity equation given in Horner et al. (1995) has the following form.

$$\frac{\partial C}{\partial t} + \nabla \cdot (vC) - D \nabla^2 C = 0$$  \hspace{1cm} (4.10)

Equation (4.10) for the cylindrical coordinates can be written as follows, as given in Horner et al. (1995).

$$v_r \frac{\partial C}{\partial r} + v_z \frac{\partial C}{\partial z} = D \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial C}{\partial r} \right) + \frac{\partial^2 C}{\partial z^2} \right]$$  \hspace{1cm} (4.11)

The following boundary conditions, as given in Horner et al. (1995), are used in solving Equation 4.11. Similar boundary conditions are also used by Haun and Hammer (2009)
for solving their transport-reaction model. For the present analysis, first order kinetics (B.C. 4 below) was used to define the reaction between ligand-conjugated particles and the receptors on the vessel wall. Thus,

B.C. 1: Boundary condition for center of the vessel: at \( r = 0, \frac{\partial C}{\partial r} = 0 \)

B.C. 2: Inlet boundary condition: at \( z = -L, C = C_0 \)

B.C. 3: Outlet boundary condition: at \( z = L, \frac{\partial C}{\partial z} = 0 \)

B.C. 4: Reactive wall boundary condition: at \( r = R_z, -D \frac{\partial C}{\partial r} = K_A C \), a first-order reaction is considered between ligands on the particle and receptors on the wall.

Before solving for concentration profiles and other analyzes, Equation 4.11 is transformed into a non-dimensional form using Haun and Hammer approach (2009), as given in the following equation, Equation 4.13. For this purpose, characteristic radial and axial velocities are used. The radial characteristic velocity is defined in the following equation, as given in Horner et al. (1995):

\[
V = v_{z\text{max}} \frac{R_z}{L} \tag{4.12}
\]

\( v_{z\text{max}} \) is the characteristic axial velocity defined by Equation 4.7, which is different from the characteristic axial velocity defined in Horner et al. (1995). Hence, the convective mass transport equation takes the following form.

\[
\bar{v}_r \frac{\partial C}{\partial r} + \bar{v}_z \frac{\partial C}{\partial z} = -\frac{1}{PeGe} \left( \frac{1}{R} \frac{\partial}{\partial R} \left( R \frac{\partial C}{\partial R} \right) \right) + Ge^2 \frac{\partial^2 C}{\partial z^2} \tag{4.13}
\]
Where, $Pe$ is the Peclet number and $Ge$ is the dimensionless characteristic length, $Pe = \frac{v_{z\text{max}} R_z}{D}$ and $Ge = \frac{R_z}{L}$.

Non-dimensional forms of velocity profiles given in Equations 4.6 and 4.9 are obtained by dividing them by $v_{z\text{max}}$.

Dimensionless forms of the boundary conditions are also obtained as given below, using the Haun and Hammer (2009) approach.

B.C. 1: at $\bar{r} = 0$, $\frac{\partial \bar{C}}{\partial \bar{r}} = 0$

B.C. 2: at $\bar{z} = -1$, $\bar{C} = 1$

B.C. 3: at $\bar{z} = 1$, $\frac{\partial \bar{C}}{\partial \bar{z}} = 0$

B.C. 4: at $\bar{r} = 1$, $\frac{\partial \bar{C}}{\partial \bar{r}} = -D_a \bar{C}$

Where,

$$D_a = \frac{K_A R_z}{D}$$

is the Damkohler number,

$D$ is particle diffusion coefficient given by following the Stokes-Einstein equation (Haun and Hammer 2008)

$$D = \frac{k_B T}{6\pi R_p}$$  \hspace{1cm} (4.13a)

Where $k_B T$ is thermal energy and $R_p$ is particle radius.
Solution strategy

A numerical approach was used to solve the model equations developed here, which is different from the approach used by Horner et al. (1995). For solving the model developed in the present study, the assumptions used by Horner et al. (1995) were used here. These were: the flow is laminar and incompressible, no slip boundary condition used at the wall, particle suspension is dilute, and lubrication theory applies where the vessel radius and stenosis height was very small compared to vessel length. To obtain concentration profiles and hence the conversion for particle adhesion along the length of the aorta, the two-dimensional space shown in Fig. 4.1 was discretized into a two dimensional mesh of grid points (Fig. 4.2) spaced at lengths equal to $\Delta z$ and $\Delta r$ in the $z$- and $y$-directions respectively. Equation 4.13 was converted into a finite difference scheme using backward difference formula for the first order derivatives and central difference formula for the second order derivatives (Chapra and Canale 1988). The concentration at each node point in the two-dimensional mesh shown in Fig. 4.2 was defined by following finite difference equation, Equation 4.14, which was obtained from Equation 4.13. A MATLAB code was developed to simultaneously solve the flow equations and the mass transport equations (Equations 4.1 through 4.13) iteratively with appropriate boundary conditions for each node point in the two-dimensional space shown in Fig. 4.2. A typical MATLAB code is given in Appendix II. The values for the parameters used in this simulation are given in Table 4.1.

$$
\overline{C}_{j,k} = \frac{1}{P_i} \left( P_2 C_{j,k+1} + P_3 C_{j,k-1} + P_4 C_{j+1,k} + P_5 C_{j-1,k} \right) \quad (4.14)
$$
Where,

\[ P_1 = \frac{\bar{v}_r}{\Delta r} + \frac{\bar{v}_z}{\Delta z} + \frac{1}{PeGe\Delta r^2} + \frac{2Ge}{Pe\Delta z^2} \]  \hspace{1cm} (4.15)

\[ P_2 = \frac{1}{PeGe\Delta r^2} \]  \hspace{1cm} (4.16)

\[ P_3 = \frac{\bar{v}_r}{\Delta r} \]  \hspace{1cm} (4.17)

\[ P_4 = \frac{Ge}{Pe\Delta z^2} \]  \hspace{1cm} (4.18)

\[ P_5 = \frac{\bar{v}_z}{\Delta z} + \frac{Ge}{Pe\Delta z^2} \]  \hspace{1cm} (4.19)

A conversion for particle adhesion was calculated from final concentrations at each point along the length of the aorta as defined in the following equation.

\[ (Conversion)_z = \frac{(Particle \ Flux)_{Inlet} - (Particle \ Flux)_z}{(Particle \ Flux)_{Inlet}} \]  \hspace{1cm} (4.20)
Figure 4.2: Finite difference scheme for the solution of convective–diffusive transport equation.

Two dimensional space between the center of the vessel and the vessel wall, shown in Fig. 4.1, is discretized using a finite difference scheme. Each node in this scheme is represented by a difference equation given by Equation 4.15 that calculates particle concentration at that node from the concentration at the surrounding nodes. Appropriate boundary conditions are applied for the nodes at the boundaries of the two-dimensional space.
### Table 4.1

Values of the parameters used in the mathematical simulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of mouse aorta (L)</td>
<td>5x10^{-2} m</td>
<td>(Huo et al. 2008)</td>
<td>5% of this assumed to be covered by plaque and used for analysis</td>
</tr>
<tr>
<td>Particle diameter (Dp)</td>
<td>1x10^{-8} m</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Average Mouse aorta diameter (Dv)</td>
<td>1.25x10^{-3} m</td>
<td>(Huo et al. 2008)</td>
<td>Uniform average diameter considered for present analysis.</td>
</tr>
<tr>
<td>First order rate constant ($K_s$)</td>
<td>1x10^{-9} m^{-1}</td>
<td>(Haun and Hammer 2008)</td>
<td>-</td>
</tr>
<tr>
<td>Average flow rate in mouse aorta and its branches (Q)</td>
<td>1.2x10^{-10} m^{3}/s</td>
<td>(Huo et al. 2008)</td>
<td>-</td>
</tr>
</tbody>
</table>
Results

Presence of a plaque affects axial and radial velocity profiles

Plaque formation due to atherosclerosis on the luminal surface causes constriction of the smooth and uniform vessel lumen. As shown in Fig. 4.1, for computational simplicity, this constriction was modeled as a bell shaped axisymmetric stenosis given by Equation 4.1 and studied its effect on velocity profiles within the plaque region. The results are shown in Fig. 4.3. As shown in Fig. 4.3A, the axial velocity is highest at the stenosis center \( z = 0 \) and decreases away from the stenosis center. This kind of an axial flow profile would be expected since the area available for flow at the stenosis center is lowest compared to the area available for flow in the rest of the vessel. Fig. 4.3B shows radial velocity profiles in the stenosed region of the aorta which would be absent in a non-stenosed aorta. As can be seen from Fig. 4.3B, the radial velocities in the region between the vessel entrance and stenosis center are negative which means they are directed away from the vessel wall. On the other hand, radial velocities in the region between the stenosis center and the vessel exit are positive meaning they are directed towards the vessel wall. Since the axial and radial velocities are normalized to the maximum axial velocity, it can also be inferred from the data in Fig. 4.3 that the axial velocities are several orders of magnitude greater than the radial velocities.
Figure 4.3: Velocity profiles in a plaque bearing aorta.

Velocity profiles are calculated along the radial and axial positions. A) Axial and B) radial velocity in the radial direction at various positions along the length of the aorta at $Pe = 1 \times 10^3$, $Da = 0.2$ and $\bar{H} = 0.1$. 
**Particle conversion along the length of the aorta increases with increasing stenosis height**

Various processes within the plaque region can alter the reaction kinetics at the endothelial surface of the aortic wall. These include convection and diffusion of particles to and away from the wall. As discussed above and shown in Fig. 4.3, results from the simulation indicated that the presence of a plaque causes substantial changes in flow profiles along the length of the aorta in the plaque region. These changes could potentially cause changes in the particle convection profiles that would normally exist in a non-stenosed aorta. Thus, to delineate the effects of these changes on particle adhesion to the wall, the effect of various stenosis heights on the conversion for the adhesion of particles was studied using the model developed here. For this part of the study, Pe and Da were held constant and the height of the stenosis was varied. Results are shown in Fig. 4.4. It is interesting to see that the conversion increases with increasing stenosis height.

Additionally, for a given conversion profile (Fig. 4.4), a lower conversion is observed at the stenosis center compared to a higher conversion in the rest of the plaque region. This difference seems to become more prominent at greater constriction (i.e. higher stenosis height). Typical concentration profiles within an aorta for a stenosis of height $H = 0.1$ are shown in Fig. 4.5. As we can see, for a given location along the length of the aorta, the concentration of particles is highest at the center of the vessel ($r = 0$) and decreases as we move towards the vessel wall (Fig. 4.5A). Similarly, for a given location along the radius, particle concentration is highest near the vessel entrance ($z = -X_0$) and decreases as we move towards the exit (Fig. 4.5B). This suggests that the particles are
taken up at the wall at a rate which is defined by a first order reaction between ligands on the particles and receptors on the wall given by B.C. 4 above.

Figure 4.4: Increase in stenosis height causes an increase in conversion for particle adhesion within the plaque region.

Effect of stenosis height on conversion for particle adhesion along the length of the aorta: Non-dimensional $\tilde{H}$ is varied from $\tilde{H} = 0$ to $\tilde{H} = 0.3$ while Peclet number (Pe) and Damkohler number (Da) are held constant at $\text{Pe} = 1 \times 10^3$ and $\text{Da} = 0.2$. Conversion is plotted against non-dimensional position along the length of the aorta.
Figure 4.5: Typical concentration profiles within the plaque region of the aorta.

Concentration profiles are calculated for $Pe = 1 \times 10^3$, $Da = 0.2$ and $\bar{H} = 0.1$. A) Concentration profiles in radial direction at various positions along the length of the aorta and B) Concentration profiles in $z$-direction at various positions along the radius.
Particle conversion along the length of the aorta increases with increasing Damkohler number and decreases with increasing Peclet number

Results from Figs. 4.3 through 4.5 indicated that presence of a stenosis causes changes in flow profiles and affects conversion for particle adhesion. In addition to the shape of aortic lumen, other parameters intrinsic to the system can affect the particle adhesion profiles. These include the Peclet and Damkohler numbers (Pe and Da respectively). These are lumped parameters that allow us to study the effect of two parameters simultaneously. For example, the Peclet number is a ratio of the convective and diffusive forces and the Damkohler number is a ratio of the reactive to the diffusive force. In the numerical solution of the model, when the effect of the Peclet number on conversion was studied, Da and stenosis height are held constant and when the effect of the Damkohler number on conversion was studied, Pe and stenosis height were held constant. The results are shown in Fig. 4.6. As shown in Fig 4.6A, Pe was varied from $1 \times 10^3$ to $4 \times 10^3$ (the range based on calculations using values in Table 4.1), and an increase in Pe caused a decrease in conversion. Fig. 4.6B shows results form the simulation when Da was varied from 0.05 to 0.8 (the range based on calculations using values in Table 4.1). An increase in Da caused an increase in conversion. Also, when the results from Fig. 4.6A and 4.6B are compared for conversion, it can be observed that the change in conversion caused by doubling Pe was greater than that caused by doubling Da.
Figure 4.6: Conversion for particle adhesion increases with an increase in Damkohler number and decreases with an increase in Peclet number.

Effect of Peclet number (Pe) and Damkohler number (Da) on conversion for particle adhesion. A) Effect of Pe on conversion, Da is held constant at Da=0.2 and Pe varied from $1 \times 10^3$ to $4 \times 10^3$. B) Effect of Da on conversion, Pe is held constant at Pe=$1 \times 10^3$ and Da varied from 0.05 to 0.8. In both cases, non-dimensional stenosis height is $\bar{H} = 0.1$ and the conversion is plotted against non-dimensional position along the length of the aorta.
Discussion

In this chapter, a mathematical analysis of particle adhesion to a plaque is presented. A two-step approach is used in studying particle adhesion to a plaque region within the aorta. Firstly, velocity profiles are generated within the stenosed region of the aorta. Secondly, the effect of velocity profiles on particle adhesion is studied by using the generated velocity profiles in the convective-diffusive mass transport model. In the model development, a simplified bell-shaped axisymmetric model is assumed for constriction.

The fluid dynamic analysis revealed that the axial and radial velocities are a function of the position along the length of the aorta. Axial velocity is highest at the stenosis center (Fig. 4.3 A) while radial velocity changes direction at the stenosis center (Fig. 4.3B). Changing direction of the radial component of the velocity may increase or decrease particle convection to or away from the vessel wall. The contribution of the radial velocity component in particle convection to or away from the wall would depend on its magnitude which in turn would depend on the extent of stenosis. Since, the model developed here assumes a mildly stenosed vessel in order to safely apply lubrication theory for the determination of velocity profiles as suggested in Horner et al. (1995), the radial component of the velocity would be several orders of magnitude smaller than the axial component, as also seen from Figs. 4.3A and 4.3B. Indeed, when the analysis was performed by excluding the radial component of the velocity, no significant change in conversion was observed (results not shown). Thus the effect of radial velocity on particle convection may be dwarfed by the effect of axial velocity.
When conversion at different stenosis heights was compared (Fig. 4.4), we observed that an increase in constriction led to an increase in conversion. In other words, when stenosis height increases, the particles within the plaque region of the aorta have less distance to travel to the wall and hence conceivably more chances to interact with the wall in a given amount of time as opposed to the particles within a non-plaque region of the aorta.

Diffusion and convection are the major processes carrying particles to the wall where they can interact with the receptors on the surfaces and become bound. It is important to determine which of the two processes is dominating in the present analysis since presence of a stenosis may impact their effect as well. It was learnt from the results shown in Fig. 4.6 that the Peclet number has a greater effect on conversion compared to the Damkohler number when the same orders of magnitude change is considered for the Peclet and Damkohler numbers. The Peclet number dictates the particle residence time within the plaque region of interest, hence, an increase in Pe means a decrease in residence time and thus less time for particles to interact with the vessel wall. The Damkohler number, on the other hand, has an opposite, although less pronounced effect, on conversion. As can be seen from Fig. 4.6B, an increase in Da leads to an increase in conversion, for example, due to an increase in reaction rate for a constant value of diffusion coefficient.

From the above analysis, it is clear that both the Peclet and Damkohler numbers have a substantial effect on conversion. In addition to convective and reactive forces, these non-dimensional parameters are also affected by the value of diffusion coefficient.
In the present analysis, the diffusion coefficient is defined by Equation (4.14A), which is a function of particle size. Thus, reducing particle size would increase particle diffusion and hence potentially affect conversion by affecting the Peclet and Damkohler numbers. This supports previous observations that particle size has a substantial effect on adhesion (Shinde Patil et al. 2001; Tees et al. 2002).

The conversion profiles obtained in the present analysis are similar in nature as the ones obtained in the analysis by Horner et al. (1995) with some differences. For example, in Figs. 7, 8 and 9 from Horner et al. (1995), it can be seen that the LDL conversion is minimal in the entrance region of the vessel and then it sharply increases near the stenosis center. They attributed this behavior to radial velocity. In the present analysis, a significantly smooth conversion profile was observed. This difference between the two studies could be attributed to the differences in the stenosis model. In the present study, a stenosis geometry which follows a smooth bell-shaped distribution was used whereas Horner et al. (1995) used a discontinuous function for their stenosis geometry.

In summary, the simplified mathematical analysis presented here indicates that there is interplay between various parameters and processes that exist within the plaque region and that these parameters and processes have substantial effects on particle adhesion. Thus, the simplistic approach used in the present mathematical analysis gives useful information in terms of the adhesion behavior of particles within a plaque region of the aorta.
CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

The main objective of this dissertation was to develop novel therapeutic and diagnostic approaches utilizing inflammatory markers of atherosclerosis. The studies presented in the previous chapters (Chapters 2, 3 and 4) are a step in this direction.

In Chapter 2, a ligand conjugated polymeric particle system was developed to target sites of atherosclerosis. Vascular cell adhesion molecule-1 (VCAM-1) was chosen as the target molecule since it is highly expressed at sites of atherosclerosis (Cybulsky et al. 2001; Ley and Huo 2001; Nakashima et al. 1998). Although this kind of site specific targeting approach has been used by other researchers to target particles to various models of inflammation in vivo (Sakhalkar et al. 2003; Sakhalkar et al. 2005), such an approach has not been tested in the complex disease of atherosclerosis until recently. As discussed in Chapter 2, when the results were compared to the results from microvasculature targeting studies by Sakhalkar et al. (2003) and Sakhalkar et al. (2005), no substantial differences were found in selectivities and ligand efficiencies for overall particle adhesion. On the other hand, the selectivities and ligand efficiencies obtained for particle adhesion to the femoral artery were ~1.1 and ~7.8 respectively, which are quite different from those for the aorta in the present study (selectivity as high as 5 and ligand efficiency as high as 32). Additionally, when the data from particle adhesion to the aorta was carefully analyzed, it was observed that the particle adhesion was non-uniform within a plaque region (Fig. 2.5) and also exhibited a functional dependence on the
location along the length of the aorta (Fig. 2.6). Combined the results in Chapter 2 indicate that the polymeric particle system developed here is a promising approach for targeting specific sites (e.g. plaques) in atherosclerosis.

Although the targeting approach developed in Chapter 2 indicates that it will work in the complex model of atherosclerosis, substantial improvements are warranted for further enhancing the performance of such systems. Some of the important areas of improvements are outlined in Chapter 2. In addition to those, a detailed study would be required that will probe the feasibility of mimicking the leukocyte adhesion more closely. Some aspects of such a study would include 1) the use of multiple markers of atherosclerosis, an approach similar to recently published studies (Eniola and Hammer 2005; Eniola et al. 2003; Ferrante et al. 2009), 2) the use of nanoparticles as opposed to micron-sized particles that might help improve the particle circulation times and 3) the use of VLA-4 as a VCAM-1 ligand instead of an antibody to VCAM-1 since VLA-4 is the original ligand involved in leukocyte-endothelium interactions. These modifications to the targeting system developed in Chapter 2 could in theory enhance in vivo performance.

With the development of a successful particle based targeting system for atherosclerosis, it was logical to also explore the development of a diagnostic tool to effectively detect the presence of atherosclerosis. This was the focus of Chapter 3 wherein a real-time PCR based system was developed to detect activated endothelial cells from whole blood samples. In Chapter 3 a simple assay was developed where vascular cell adhesion molecule (VCAM-1) and melanoma cell adhesion molecule (MCAM) were
used as markers for inflamed endothelial cells. Previous studies have shown that MCAM and other endothelial markers can be used to detect endothelial cells in protein based detection systems which include magnetic bead separation and flow cytometry (Clarke et al. 2008; Duda et al. 2007; Goon et al. 2006; Widemann et al. 2008). A preliminary literature survey indicated that the study presented in Chapter 3 is the first time a dual marker approach was developed using a real-time quantitative PCR based technique for detecting activated endothelial cells from whole blood. The assay developed in Chapter 3 may serve as a promising diagnostic/prognostic approach for detecting activated endothelial cells in blood samples from atherosclerotic patients.

The real-time quantitative PCR assay developed in Chapter 3 needs to undergo further improvements and optimizations before it can be used in a clinical setting. As a next step in the development of the assay, clinical blood samples from atherosclerotic patients should be tested. For this purpose, the blood samples can be drawn either from the general circulation or from a specific vascular site (e.g. a plaque). Thus, the assay can be tested for its effectiveness in predicting the presence of circulating endothelial cells (CECs) in atherosclerotic blood samples. Since the typical circulating endothelial cell (CEC) count in atherosclerotic blood samples is ~1-5 CECs/ml (Makin et al. 2004), the assay needs to be sensitive enough to effectively detect a single endothelial cell per milliliter. The sensitivity of the current assay may be improved by enriching the RNA of interest from the total RNA obtained from the blood sample. This can be achieved in two ways, one by purification of mRNA from the total RNA using magnetic beads conjugated with oligo-dT or by purification of the target mRNA (e.g. VCAM-1 or MCAM mRNA).
from the total RNA using magnetic beads conjugated with primers specific to the gene of interest. Oligo-dT or primer conjugated magnetic beads can be tailor-made or obtained commercially. Thus, the future work in the diagnostic/prognostic assay development for atherosclerosis should be designed to improve the assay sensitivity as it applies to the clinical samples.

Chapter 4 is a logical progression of the study presented in Chapter 2. The particle system developed in Chapter 2 indicated that there was an interesting pattern to the adhesion profiles within the aorta. In an in vivo model of atherosclerosis, the presence of plaque may substantially influence particle adhesion. In fact, the presence of plaque can be seen as the major difference between the studies presented in Chapter 2 and the previous in vivo targeting studies (Sakhalkar et al. 2003; Sakhalkar et al. 2005). To test if the presence of plaque caused any substantial changes in the adhesion profiles, a mathematical analysis was performed in Chapter 4. A simplified mathematical model was developed for studying the adhesion of particles to a plaque whose structure was approximated by an axisymmetric bell-shaped stenosis. Results from this analysis indeed indicated that the presence of a plaque substantially changed the adhesion profiles within a plaque region. This study also determined the effect of other system parameters such as the Peclet number and the Damkohler number and found that they too have substantial effects on particle adhesion.

Although the simplified mathematical analysis presented in Chapter 4 gives insightful information on particle adhesion behavior within the plaque regions of a vessel affected by atherosclerosis, further modifications can be made to the mathematical model
to closely approximate the *in vivo* conditions. Some of the modifications that can be introduced in the current model include 1) the use of an asymmetric plaque geometry that closely resembles the shape and size of a plaque observed *in vivo*, 2) the use of a non-uniform distribution of receptors (e.g. VCAM-1) on the endothelial surface by defining receptor concentration as a function of \( z \), and 3) the use of a comprehensive reaction model to define the interactions between the ligand conjugated particles and the receptors on the endothelium. Further, the results from the mathematical simulation can be compared to experimental outcomes and specific experiments may be designed to obtain accurate *in vivo* parameters for the mathematical analysis. Thus, the mathematical analysis developed in Chapter 4 can be further improved by incorporating various modifications suggested above.

In summary, this dissertation has demonstrated that using molecular markers of atherosclerosis, novel therapeutic and diagnostic approaches can be developed that may substantially impact how atherosclerosis is diagnosed and treated in the near future.
REFERENCES


Bardin N, Reumaux D, Geboes K, Colombel JF, Blot-Chabaud M, Sampol J, Duthilleul P, Dignat-George F. 2006. Increased expression of CD146, a new marker of the


**APPENDIX I: NOMENCLATURE AND ABBREVIATIONS**

- An overbar, represents a non-dimensional parameter

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2H$</td>
<td>Height of stenosis at the center of plaque</td>
</tr>
<tr>
<td>$2X_0$</td>
<td>Spread of the stenosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE$^{-/-}$ mouse</td>
<td>Mouse lacking Apolipoprotein E gene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Particle concentration</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CEC</td>
<td>Circulating endothelial cell</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>D</td>
<td>Particle diffusivity</td>
</tr>
<tr>
<td>Da</td>
<td>Damkohler number</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>$D_p$</td>
<td>Particle diameter</td>
</tr>
<tr>
<td>ECAI</td>
<td>Endothelial cell activation index</td>
</tr>
<tr>
<td>ECAMs</td>
<td>Endothelial cell adhesion molecules</td>
</tr>
<tr>
<td>ELAM</td>
<td>Endothelial-leukocyte adhesion molecule</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EVMR</td>
<td>Endothelial VCAM-1 to MCAM ratio</td>
</tr>
<tr>
<td>Ge</td>
<td>Dimensionless characteristic length</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>KA</td>
<td>First order adhesion rate constant</td>
</tr>
<tr>
<td>kB</td>
<td>Bolzmann constant</td>
</tr>
<tr>
<td>L</td>
<td>Length of the vessel</td>
</tr>
<tr>
<td>LEAP</td>
<td>Leukocyte-endothelial cell adhesive particle</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MCAM</td>
<td>Melanoma cell adhesion molecule</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical density</td>
</tr>
<tr>
<td>P</td>
<td>Pressure</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pe</td>
<td>Peclet number</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet/endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly (lactic acid)</td>
</tr>
<tr>
<td>PSA</td>
<td>Poly (sebacic acid)</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>Q</td>
<td>Volumetric flow rate</td>
</tr>
<tr>
<td>qRT qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>r</td>
<td>Radius of the vessel</td>
</tr>
<tr>
<td>R₀</td>
<td>Radius of the vessel at stenosis entrance</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rₚ</td>
<td>Particle radius</td>
</tr>
<tr>
<td>Rₚz</td>
<td>Radius of the vessel as a function of z</td>
</tr>
<tr>
<td>T</td>
<td>Physiological temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor-necrosis factor-α</td>
</tr>
<tr>
<td>V</td>
<td>Characteristic radial velocity</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>vᵣ</td>
<td>Radial velocity</td>
</tr>
<tr>
<td>vᵣz</td>
<td>Axial velocity</td>
</tr>
<tr>
<td>YG</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>z</td>
<td>Position along the vessel (aorta) length</td>
</tr>
<tr>
<td>α-E-selectin</td>
<td>Monoclonal antibody to E-selectin</td>
</tr>
<tr>
<td>α-ICAM-1</td>
<td>Monoclonal antibody to ICAM-1</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>α-VCAM-1</td>
<td>Monoclonal antibody to VCAM-1</td>
</tr>
</tbody>
</table>
APPENDIX II: MATLAB CODE TO CALCULATE CONVERSION AT DIFFERENT STENOSIS HEIGHTS

PROGRAM TO STUDY THE EFFECT OF STENOSIS HEIGHT ON CONVERSION FOR PARTICLE ADHESION TO AN ATHEROSCLEROTIC PLAQUE.

This Program iteratively calculates conversion for particle adhesion at various stenosis height. Axial and radial velocity profiles are calculated and used in the mass transfer equation. Each node point in a two-dimensional grid space is defined by a finite difference equation. The concentration profiles generated from the simulation are used to calculate conversion at various locations along the length of aorta.

Following parameter values are used for this program, Pe = 1e3 and Da = 0.2. Stenosis height is varied from 0 to 30% of the radius of the aorta.

clear all;
cic;
% ------------------------------------------------------------------------
% Constants and parameters used in the program
% ------------------------------------------------------------------------
Dp = 1e-6; % Particle diameter in meters
Rp = Dp/2; % Particle radius in meters
kb = 1.38065e-23; % Boltzman constant in J/K
T = 310; % Physiological temperature in kelvin
mu = 0.798e-3; % N s/m2 (or Pa s) at 30 degree C
CC0 = 6.67e12; % #/m3 of particles circulating in mouse blood at steady state
D_vessel = 1.25e-3; % Mouse aorta diameter
R0 = (D_vessel)/2;
Ka = 1e-9; % m/s from Haun and Hammer 2008
D = kb*T/(6*pi*mu*Rp);
Aorta_length = 5e-2; % Total length of mouse aorta in meters
L = 0.05*Aorta_length; % Total length of aorta under analysis, plaque situated in the center
Q = 1.22e-8; % Flow rate, m3/s
Pe = 1e3;
Da = 0.2;
% ------------------------------------------------------------------------
% Variables to define 2D grid space and grid points
% ------------------------------------------------------------------------
jN=101;
kN=51;
DIV_j=50;
DIV_k=50;
% ------------------------------------------------------------------------
% Creation of matrix for the values of the variables to be stored
% ------------------------------------------------------------------------
CC_New = zeros(jN,kN);
CC = zeros(jN,kN);
r_dash = zeros(jN,kN);
z_dash = zeros(jN,kN);
Rz_dash = zeros(jN,kN);
Vz_dash = zeros(jN,kN);
Vz = zeros(jN,kN);
\%
% Iterative program
%
\H_{\text{fraction}}=0.0; \% Fraction of radius for stenosis height
\begin{verbatim}
for \text{stn}=1:1:4 %Loop to vary stenosis height
  \H = \R_0*\H_{\text{fraction}};
  \X_0 = \L/2; \% Total spread of the stenosis is 2\X_0
  \X_0_{\text{dash}} = 2*\X_0/\L; \%
  \H_{\text{dash}} = \H/\R_0; \%Non-dimensional stenosis height
  \text{iterations}=0;
  \text{iter}=1;
  \text{while(iter>0)}% Loop to check a predefined error tolerance using variable "iter"
    \text{iter}=0;
    \text{z_{start}}=-\L;
    \L_{\text{div}} = \L/\text{DIV}_j;
    \text{for j}=1:1:jN % Length loop
      \text{r_{start}}=0;
      \text{for k}=1:1:kN % Radius loop
        \r(j,k)= \r_{\text{start}};
        \z(j,k)= \z_{\text{start}};
        \z_{\text{dash}}(j,k)=\z(j,k)/\L;
        \R_{\text{z-dash}}(j,k)=\R_{\text{z}}(j,k)/\R_0;
        \V_{\text{z}}(j,k)=2*\Q/(\pi*\R_{\text{z}}(j,k)^2)*(1-\r(j,k)^2/\R_{\text{z}}(j,k)^2);
        \V_{\text{z-max}} = 2*\Q/(\pi*(\R_0-2*\H)^2);
        \V_{\text{z-dash}}(j,k)=\V_{\text{z}}(j,k)/\V_{\text{z-max}};
        \r_{\text{dash}}(j,k)=\r(j,k)/\R_{\text{z}}(j,k);
        \V_{\text{r}}(j,k) = \Q*\H/(2*\R_{\text{z}}(j,k)^2)*\sin(\pi*\z(j,k)/(2*\X_0))\times(\r_{\text{dash}}(j,k)-\r_{\text{dash}}(j,k)^3);\V_{\text{r}}(j,k)=0;
        \V_{\text{r-dash}}(j,k) = \V_{\text{r}}(j,k)/\V_{\text{z-max}};
        \R_{\text{div}}=\R_{\text{z}}(j,k)/\text{DIV}_k;
        \text{Ge} = \R_{\text{z}}(j,k)/\L;
        \text{delta_r}=1/\text{DIV}_k; \%Non-dimensional spacing in y-direction
        \text{delta_z}=1/\text{DIV}_j; \%Non-dimensional spacing in z-direction
        \P_1=\V_{\text{r-dash}}(j,k)/\delta_{\text{r}}+\V_{\text{z-dash}}(j,k)/\delta_{\text{z}}+1/(\text{Pe}*\text{Ge}*\delta_{\text{r}}^2)+2*\text{Ge}/(\text{Pe}*\delta_{\text{z}}^2);
        \P_2=1/(\text{Pe}*\text{Ge}*\delta_{\text{r}}^2);
        \P_3=\V_{\text{r-dash}}(j,k)/\delta_{\text{r}};
        \P_4=\text{Ge}/(\text{Pe}*\delta_{\text{z}}^2);
        \P_5=\V_{\text{z-dash}}(j,k)/\delta_{\text{z}}+\text{Ge}/(\text{Pe}*\delta_{\text{z}}^2);
      \end{verbatim}
\end{verbatim}
%Boundary condition at the center of the vessel
\begin{verbatim}
if(k==1&j>1&j<jN)
  \text{CC}_{\text{New}}(j,k) = (1/\P_1)*((\P_2+\P_3)*\text{CC}(j,k+1)+((\P_4+\P_5)*\text{CC}(j+1,k));
else
  \text{CC}_{\text{New}}(j,k) = (1/\P_1)*((\P_2+\P_3)*\text{CC}(j,k+1)+\P_4*\text{CC}(j+1,k)+\P_5*\text{CC}(j-1,k));
end
\end{verbatim}
%Boundary condition at the vessel wall
\begin{verbatim}
if(k==kN&j>1&j<jN)
  \text{CC}_{\text{New}}(j,k) = 1/(\P_1+2*\P_2*\delta_{\text{r}}*\text{Da})*((\P_2+\P_3)*\text{CC}(j,k-1)+((\P_4+\P_5)*\text{CC}(j-1,k));
else
end
\end{verbatim}
CC_New(j,k) = \frac{1}{P1+2*P2*\delta_r*Da}*((P2+P3)*CC(j,k-1)+P4*CC(j+1,k)+P5*CC(j-1,k));

% Concentration at internal node points
if(k>1\&\&k<kN\&\&j>1\&\&j<jN)
    CC_New(j,k) = \frac{1}{P1}*(P2*CC(j,k+1)+P3*CC(j,k-1)+P4*CC(j+1,k)+P5*CC(j-1,k));
end

% Boundary condition at the inlet
if(j==1\&\&k>=1)
    CC_New(j,k) = 1;
end

% Boundary condition at the exit
if(j==jN\&\&k>=1\&\&k<kN)
    if(k==1)
        CC_New(j,k) = 1/P1*((P2+P3)*CC(j,k+1)+(P4+P5)*CC(j-1,k));
    elseif(k==jN)
        CC_New(j,k) = 1/P1*(P2*CC(j,k+1)+P3*CC(j,k-1)+(P4+P5)*CC(j-1,k));
    end
end

CC_Err = abs(CC_New(j,k)-CC(j,k)); % Error from previous iteration
if(CC_Err<1e-3)
    iter=iter+0;
else
    iter=iter+1;
end
CC(j,k) = CC_New(j,k);
r(j,k) = r(j,k)+R_div;
r_start = r(j,k);
X(j,k) = 1-CC(j,kN);
end

Vol = R_div*R_div*L_div;
Ax = R_div*R_div; % cross-sectional area of one grid
Flux(j) = CC0*sum(CC(j,1:kN))*Vol/Ax;
Conv(j) = (Flux(1)-Flux(j))/Flux(1);
if(stn==1)
    CV1 = [Conv];
end
if(stn==2)
    CV2 = [Conv];
end
if(stn==3)
    CV3 = [Conv];
end
if(stn==4)
    CV4 = [Conv];
end
zz(j)=z_dash(j,k);
z(j,k) = z(j,k)+L_div;
z_start = z(j,k);
H_fraction=H_fraction+0.1;
end

% Conversion data transferred to MSExcel for further analysis
% ----------------------------------------------------------------------------------------------
data = [zz;CV1;CV2;CV3;CV4];
d = data';
DataTitle = {'zz','H-dash= 0.0','H-dash= 0.1','H-dash= 0.2','H-dash= 0.3'};
xlswrite('EffectOfStenosis.xls',DataTitle,'EffectOfStenosis','A1')
xlswrite('EffectOfStenosis.xls',d,'EffectOfStenosis','A2')
APPENDIX III: REPRINT PERMISSIONS FOR THE MATERIAL USED IN

THIS DISSERTATION

1) Permission to use a published figure from Nature:

<table>
<thead>
<tr>
<th>NATURE PUBLISHING GROUP LICENSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TERMS AND CONDITIONS</td>
</tr>
<tr>
<td>Jan 30, 2010</td>
</tr>
</tbody>
</table>

This is a License Agreement between Sudhir P Deosarkar ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number: 2359021068964
License date: Jan 30, 2010
Licensed content publisher: Nature Publishing Group
Licensed content publication: Nature
Licensed content title: Inflammation in atherosclerosis
Licensed content author: Peter Libby
Volume number: 0
Issue number: 0
Pages: 0
Year of publication: 2002
Portion used: Figures / tables
Number of figures / tables: 1
Requestor type: Student
Type of Use: Thesis / Dissertation
Billing Type: Invoice
Company: Sudhir P Deosarkar
Billing Address: 006, Konnaker Research Center, The Ridges
Ohio University
Athens, OH 45701
United States
Customer reference info: 0.00 USD

Total: 0.00 USD
2) **Permission to use a published figure from Elsevier:**

**ELSEVIER LICENSE TERMS AND CONDITIONS**

Mar 04, 2010

---

This is a License Agreement between Sudhir P Deosarkar ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

**All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.**

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Elsevier Limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Registered Company Number</td>
<td>1982084</td>
</tr>
<tr>
<td>Customer name</td>
<td>Sudhir P Deosarkar</td>
</tr>
<tr>
<td>Customer address</td>
<td>006, Konneker Research Center, The Ridges</td>
</tr>
<tr>
<td></td>
<td>Athens, OH 45701</td>
</tr>
<tr>
<td>License Number</td>
<td>2362066007546</td>
</tr>
<tr>
<td>License date</td>
<td>Mar 04, 2010</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Clinica Chimica Acta</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Circulating endothelial cells: A novel marker of endothelial damage</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Uta Erbruegger, Marion Haubitz, Alexander Woywodt</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>November 2006</td>
</tr>
<tr>
<td>Volume number</td>
<td>373</td>
</tr>
<tr>
<td>Issue number</td>
<td>1-2</td>
</tr>
<tr>
<td>Pages</td>
<td>10</td>
</tr>
<tr>
<td>Type of Use</td>
<td>Thesis / Dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>Figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of Figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>Format</td>
<td>Both print and electronic</td>
</tr>
<tr>
<td>You are an author of the Elsevier article</td>
<td>No</td>
</tr>
<tr>
<td>Are you translating?</td>
<td>No</td>
</tr>
<tr>
<td>Order Reference Number</td>
<td></td>
</tr>
<tr>
<td>Expected publication date</td>
<td>Mar 2010</td>
</tr>
</tbody>
</table>
3) Permission to reuse a published Biotechnology and Bioengineering paper from John Wiley and Sons:

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 06, 2010

This is a License Agreement between Sudhir P Deosarkar ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>License Number</th>
<th>2363150341791</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Feb 06, 2010</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>John Wiley and Sons</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Biotechnology &amp; Bioengineering</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Polymeric particles conjugated with a ligand to VCAM-1 exhibit selective, avid, and focal adhesion to sites of atherosclerosis</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Deosarkar Sudhir P., Malgor Ramiro, Fu Jie, et al</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>Mar 12, 2008</td>
</tr>
<tr>
<td>Start page</td>
<td>400</td>
</tr>
<tr>
<td>End page</td>
<td>407</td>
</tr>
<tr>
<td>Type of use</td>
<td>Dissertation/Thesis</td>
</tr>
<tr>
<td>Requestor type</td>
<td>Author of this Wiley article</td>
</tr>
<tr>
<td>Format</td>
<td>Print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>Full article</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Order reference number</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>