DEVELOPMENT OF CARDIOVASCULAR NANODEVICES FOR THE DETECTION OF VULNERABLE PLAQUE

A Thesis

Presented in Partial Fulfillment of the Requirements for
the Degree Master of Science in the
Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University
2006

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ABSTRACT

The primary health problem facing Americans today is cardiovascular disease. Cardiovascular disease results in more deaths every year than the next four leading causes of death combined (cancer, respiratory diseases, accidents, and diabetes). A particularly frightening fact is that about two thirds of unexpected cardiac deaths occur without manifestation of symptoms related to heart disease. Many of these unexpected deaths occur when a vulnerable plaque ruptures, exposing prothrombotic material to the circulation, often resulting in complete and catastrophic occlusion of the blood vessel. Current medical imaging modalities (CT, MRI, angiography, etc.) can identify narrowing of the arteries, but they are all limited in the information they provide regarding the vulnerability of the plaque.

This work will present results aimed at identifying novel targeting agents to vulnerable plaque with the goal of developing a targeted contrast-enhancing nanodevice that can be used to detect vulnerable plaque before rupture. Phage displayed peptide libraries were used to probe the molecular architecture of the Watanabe heritable hyperlipidemic (WHHL) rabbit animal model of atherosclerosis. Over 200 novel peptide sequences were isolated, of which 45 were selected for synthesis and further assaying. Binding assays were conducted in several systems: lipid-loaded human aortic endothelial
cells (HAECs), WHHL rabbits, New Zealand white (NZW) rabbits (a non-diseased control animal), and ApoE knockout mice (murine model of atherosclerosis). One peptide in particular, TQTPIKHLLKE (peptide 9), was found to consistently bind plaque in the WHHL model in a dose-dependent manner. Furthermore, binding of peptide 9 to the NZW model was seen as light background binding, which did not increase with increased concentration of peptide. This indicates that peptide 9 is a potential plaque-targeting agent and should be used in future work to develop targeted image-contrast nanodevices to atherosclerotic plaque. Results for all other peptides are presented and discussed as well. Experiments to isolate the protein to which peptide 9 binds were unsuccessful, but recommendations are made that will improve the likelihood of isolating the protein.
Dedicated to
my parents,
Terry and Maryellen,
who dealt me an amazing
starting hand.
ACKNOWLEDGMENTS

It is impossible to conduct such multidisciplinary research on one's own, and for this fact I must thank many individuals who have provided support for this project. I would like to thank my adviser, Dr. Steve Lee, who was willing to let a mechanical engineer try his hand at biology in his lab and for offering unconditional support and guidance throughout my graduate studies. I must also thank Mr. John Shapiro for his immeasurable knowledge of molecular biology and for his patience in teaching me every lab technique I have learned. John is not only an excellent instructor, but also a great friend. I would be remiss if I forgot to thank the members of the research group with whom I have worked on one or more projects and who help keep the lab interesting: Theo Nicholson, Bryan Smith, Jason Sakamoto, Jonathan Pillai, Matt Keener, and Edward Eteshola. I also need to thank Dr. Nicanor Moldovan for agreeing to participate on my examination committee. As no research is possible without funding, I would like to thank the OSU Graduate School for a generous first year fellowship and the OSU Mathematics Department for their continued assistance. Finally, I would like to acknowledge all the researchers and staff at the Davis Heart and Lung Research Institute; they are the ones who help keep the research going.
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# NOMENCLATURE AND ABBREVIATIONS

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<tr>
<td>ACS</td>
<td>Acute Coronary Syndromes</td>
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<tr>
<td>AHA</td>
<td>American Heart Association</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DAB</td>
<td>Diamino Benzidine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FLAG</td>
<td>The peptide sequence, DYKDDDDK</td>
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<tr>
<td>HAECs</td>
<td>Human Aortic Endothelial Cells</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IVUS</td>
<td>Intravascular Ultrasound</td>
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<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
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<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>Near-infrared</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>OCT</td>
<td>Optical Coherence Tomography</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>TCFA</td>
<td>Thin-Cap Fibroatheroma</td>
</tr>
<tr>
<td>USPIOs</td>
<td>Ultra-small Superparamagnetic Iron Oxide (particles)</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe heritable hyperlipidemic (rabbit)</td>
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CHAPTER 1
INTRODUCTION

1.1 Cardiovascular and Coronary Heart Disease

Cardiovascular disease (CVD) includes high blood pressure, coronary heart disease, heart failure, stroke, and congenital cardiovascular defects. Preliminary mortality data show that CVD as the underlying cause of death in the United States accounted for 37.3% of all deaths in 2003 [1]. CVD has been the number one killer in the U.S. every year since 1900, except for 1918. On average CVD causes one American to die every 35 seconds, and CVD claims more lives each year than the next four leading causes of death combined (cancer, chronic lower respiratory diseases, accidents, and diabetes mellitus). The estimated costs (direct and indirect) of CVD for 2006 amount to a staggering $403.1 billion [1].

Coronary heart disease (CHD), a subset of cardiovascular diseases including myocardial infarction (MI, or heart attack) and angina pectoris (chest pain), caused 1 out of every 5 deaths in the United States in 2003, making it the single largest killer of Americans [1]. CHD comprises more than half of all cardiovascular events in men and women under age 75. One of the more disconcerting aspects of heart disease is that
about two thirds of unexpected cardiac deaths occur without prior symptoms. Clearly, there is much room for improvement in the diagnosis and therapy of CHD.

1.2 Vulnerable Atherosclerotic Plaque

Atherosclerosis is defined by the National Heart, Lung, and Blood Institute to be the hardening and narrowing of the arteries. As the name implies, atherosclerosis consists of two main components: a soft, lipid-rich atheromatous “gruel” and hard, collagen-rich sclerotic tissue [2]. Atherosclerosis is a chronic disease characterized by lipid and matrix deposition, neoangiogenesis and inflammation, vessel wall remodeling, and abnormal vasomotor regulation [3]. The arterial occlusion is caused by the slow buildup of plaque on the intimal surface of the artery. Atherosclerotic plaque can be divided into two fractions: stable and vulnerable. Generally speaking, stable plaque is less prone to rupture than vulnerable plaque. The exact morphological and pathological distinction between the two fractions is a matter currently under debate.

The history of the vulnerable plaque can be traced back to 1966 when Dr. Paris Constantinides published convincing evidence that plaque rupture was the cause of onset of most acute cardiovascular diseases [4]. Dr. Constantinides examined the coronary arteries of 22 patients with coronary artery disease. Of these 22 cases, 17 had acute coronary thrombosis. All 17 of these acute thrombi were found to originate over cracks in the intimal surface of the plaque. This observation has been confirmed by many investigators and is the basis of the current understanding that the atherosclerotic substrate causes thrombus formation in the arterial circulation. In 1984 Willerson et al. proposed the idea that the conversion of chronic to acute coronary disease occurs when
an anatomically altered plaque becomes a stimulus for platelet adhesion, aggregation, and mediator accumulation [5]. These mediators include thromboxane A₂ and serotonin, which when generated cause thrombosis and vasoconstriction.

By 1994 there was widespread recognition of the importance of plaque rupture, thrombosis, and mediator generation in the onset of cardiovascular disease [6]. Based on this knowledge, the term "vulnerable" can be used to describe a plaque that by becoming disrupted has a high likelihood of starting the adverse cascade leading to the acute coronary event [7]. This is an uncomplicated functional definition that (a) does not depend on the histology of the plaque and (b) does not indicate how such a plaque can be identified prospectively or stabilized before it causes the onset of disease. Evidence continues to suggest, but does not prove, that plaques with a large lipid core, thin cap, and increased macrophage content should be considered vulnerable plaques [6]. Plaques meeting all three of the criteria listed above can be described as inflamed thin-cap fibroatheromas (TCFAs). It is commonly assumed that TCFAs represent vulnerable plaques, but there are no prospective studies to date proving that they are at increased risk of leading to thrombosis and lesion progression. Furthermore, there appear to be plaques without these histologic features that may be associated with arterial thrombosis and should therefore be considered vulnerable [6].

While inflamed TCFAs are likely to be vulnerable, recent pathologic studies have revealed that a number of plaques causing thrombosis, hence vulnerable plaques, were not TCFAs. Farb et al. studied plaque characteristics in 50 subjects with sudden cardiac death due to coronary thrombosis [8]. In 44% of the cases the thrombosis occurred at a
site with erosion that did not occur over a thin cap and lipid pool. In fact, the plaque appeared to have increased proteoglycan content. Thus, a plaque rich in proteoglycans is a second histological type of vulnerable plaque. In addition to this type of vulnerable plaque, Virmani et al. have identified a calcified nodule as a potentially vulnerable plaque [9]. Further difficulty arises in attempting to label a TCFA as vulnerable, because it is known that plaques with a large lipid pool, thin cap, and increased macrophage content that are not disrupted can be found at autopsy. Such plaques may only cause stable angina, although their histologic features suggest they could produce an acute coronary syndrome (ACS).

The most useful definition of a vulnerable plaque would be one that can be applied to individual lesions prospectively. In 1995 Uchida et al. demonstrated that angioscopy could identify vulnerable plaque prospectively in living patients [10]. In this study the three coronary arteries of 157 patients with angina received a prospective angioscopic survey. Thirteen of these patients had a "glistening yellow plaque," shown in a separate autopsy series to represent a lipid pool with a very thin cap. During a one-year follow-up, 68% of the patients with the vulnerable glistening yellow plaque experienced an acute coronary syndrome, while only 4% of those without such a plaque experienced an event. While this observation is valuable for research purposes, it is unlikely to have much clinical utility, because angioscopy requires coronary artery occlusion (hence it is invasive and not well suited for screening), the identification of a "glistening yellow" appearance is very subjective, and angioscopy cannot detect the other
types of vulnerable plaques discussed above that are not associated with a lipid pool and a thin fibrous cap.

There are two other terms related to the vulnerable plaque that appear in the literature and require some clarification. The term "disrupted" plaque has been used in some cases to refer to a plaque that causes some degree of thrombosis, but in other cases describes only the nature of the lesion. Perhaps the term "thrombosed" plaque would be more appropriate to use to indicate the transition from a vulnerable plaque to a thrombosed plaque. In addition to these terms, the term "unstable" plaque has received highly variable usage. In some instances, unstable has been used to describe vulnerable plaques, but in other cases it refers to disrupted plaques as defined above. Clearly, there is much difficulty in defining the vulnerable plaque, but it is also evident that there appears to be some consensus as to the characteristics of the vulnerable plaque. It should be noted that vulnerable plaque is clearly a subset of atherosclerotic plaque, which is characterized by intimal thickening due to cellular and lipid accumulation.

A summary of the criteria for defining vulnerable plaque is provided in Table 1.1 [11]. The presence of one or a combination of the major factors may imply an increased risk of plaque complication leading to rupture. Plaques with active inflammation are identified by extensive macrophage accumulation. A thin cap with a large lipid core is defined to have a cap thickness of <100 μm and a lipid core comprising >40% of the total plaque volume. Plaques with endothelial denudation and superficial platelet aggregation are characterized by superficial erosion of the endothelial layer and platelet aggregation or fibrin deposition. Plaques with a fissured cap most often have involved a recent
rupture that did not result in occlusive thrombi. These may be prone to subsequent thrombosis, which may be occlusive and lead to MI [11]. Finally, on the surface of plaques with severe stenosis, shear stress imposes a significant risk of thrombosis and sudden occlusion, making these plaques inherently vulnerable.

**Major criteria**
- Active inflammation (monocyte/macrophage and sometimes T-cell infiltration)
- Thin cap with large lipid core
- Endothelial denudation with superficial platelet aggregation
- Fissured plaque
- Stenosis >90%

**Minor criteria**
- Superficial calcified nodule
- Glistening yellow appearance
- Intraplaque hemorrhage
- Endothelial dysfunction
- Outward (positive) remodeling

Table 1.1: **Criteria for defining vulnerable plaque.** Reproduced from [11].

### 1.3 Pathophysiology of Plaque Formation and Rupture

According to the American Heart Association (AHA) Committee on Vascular Lesions, atherosclerotic plaque progression can be divided into five phases and lesion types as shown in Figure 1.1. The early lesions are the so-called "fatty streaks" that appear in 65% of children between ages 12 and 14. Typically, by the third decade of life these lesions develop a cap of smooth muscle cells and collagen, thus forming a fibroatheroma. The formation of these fibrous caps is generally slow (phase 1, types 1-
III). These early atherosclerotic lesions can progress without compromising the lumen because of compensatory vascular enlargement (remodeling).

Of particular importance are the phase 2 types IV and Va lesions, because they are more susceptible to disruption and subsequent thrombosis; hence, they can be considered vulnerable. Type IV lesions have a high extracellular lipid content intermixed with fibrous tissue beneath a fibrous cap. Type Va lesions contain a larger lipid-rich core with a thin fibrous cap. Upon disruption, these lesions may lead to acute occlusive thrombosis associated with the acute coronary syndromes (phase 3 or 4). Phase 3 is characterized by a small mural thrombus, whereas phase 4 lesions consist of an occlusive thrombus. This abrupt progression of disrupted vulnerable plaques leads to the frequent complicated type VI lesion that accounts for about 75% of the patients with acute coronary syndromes. Rupture of a vulnerable phase 2 lesion need not result in ischemic sudden death; changes in the geometry of a disrupted plaque can lead to the more occlusive and fibrotic lesions characteristic of phase 5 type Vb or Vc lesions. Types Vb or Vc lesions may cause angina; however, a final occlusion may be silent or clinically unapparent due to the fact that the preceding stenosis and ischemia can stimulate the development of protective collateral circulation [12].
Figure 1.1: **Lesion morphology and phases of progression of atherosclerosis.** According to criteria set forth by the American Heart Association (AHA), plaque progression can be divided into five phases. **Vulnerable plaques**, which are typically composed of a rich lipid core separated from the lumen by a thin fibrous cap, are type IV and Va. These phase 2 plaques are particularly soft and prone to disruption and can evolve into acute phase 3 and 4 plaques, causing unstable angina, myocardial infarction, and ischemic sudden death. Reproduced from [6].

The pathogenesis of atherosclerosis contains many factors, but the two aspects considered to be of greatest importance are (a) the deposition of lipids that are the metabolized abnormally and oxidized in the vascular wall and (b) the local infiltration of leukocytes. This combination of effects creates an inflammatory environment in which the function of the endothelial cells is adversely affected, and one of the manifestations of this alteration is an increase in the potential for interaction with platelets. Platelets have been identified as a critical factor linking atherosclerosis, thrombosis, and inflammation [13].
Plaque rupture occurs when the fibrous cap is disrupted or ruptured, allowing the flowing blood to come in contact with the lipid-rich core. Figure 1.2 shows an example of a ruptured plaque leading to thrombus formation [6].

Figure 1.2: **Example of thin-cap plaque rupture.** This vulnerable plaque ruptured near the shoulder region (arrows), exposing the lipid-rich core to the flowing blood, resulting in the formation of an occlusive thrombus followed by myocardial infarction and death. The following abbreviations are used in the figure: Chol, cholesterol-rich region of plaque; Fib, fibrin; Plts, platelets. Reproduced from [6].

Thrombosis is triggered following plaque rupture when thrombogenic components of the plaque (collagen and the lipid core) are exposed to blood. The lipid core tends to be the most thrombogenic part of the plaque due to two factors: (1) the oxidized lipids contained within the core of the plaque have direct platelet activating effects, and (2) the lipid core contains a catalytically active tissue factor that activates the
extrinsic clotting cascade leading to thrombin generation and thrombus formation at sites of plaque rupture [14].

There are three major mechanical factors that determine the vulnerability of the fibrous cap to rupture: circumferential wall stress (cap fatigue), lesion characteristics (e.g., location, size, and consistency), and blood flow characteristics. It should be noted that rupture of the fibrous cap usually occurs in the shoulder regions, the weakest portion of the cap where stress is highest. Plaque disruption, however, is not a purely mechanical process; inflammation is also important. Activated inflammatory cells (macrophages, foam cells) have been detected in the disrupted areas of atherectomy specimens from patients with acute coronary syndromes [15]. These cells are capable of degrading extracellular matrix by expressing a host of matrix-degrading metalloproteinases (MMPs), which can weaken the fibrous cap, leading to increased plaque vulnerability [16].

Normal endothelium plays a pivotal role in vascular homeostasis through the balanced production of vasodilators such as nitric oxide (NO) and vasoconstrictors such as endothelin-1 (ET-1) and limits the development of atherosclerosis. The endothelium is an intrinsic determinant of plaque stability; it regulates inflammation within the intima, mediated by changes in the expression of cellular adhesion molecules and chemoattractants. The endothelium also modulates the consequences of plaque disruption through its control of vascular tone and its antithrombotic and antiplatelet functions [17].
Endothelial dysfunction is considered the earliest pathologic signal of atherosclerosis. Cardiovascular risk factors including smoking, high cholesterol, hypertension, and diabetes impair endothelial function and may trigger atherosclerosis without the need for physical endothelial injury [18]. These risk factors have been recognized to induce endothelial dysfunction by reducing the bioavailability of NO, increasing tissue ET-1 content, and activating proinflammatory signaling pathways. Furthermore, the endothelium appears to contribute to the recruitment of inflammatory cells in the arterial wall in response to oxidized LDL and changes in the structure of the plaque that can increase its vulnerability. The endothelium adjacent to a disrupted plaque is likely to exert an influence on the consequences of plaque rupture by modulating platelet aggregation, the size of the associated thrombus, and focal vasospasm [18].

Figure 1.3 shows a schematic representation of normal versus dysfunctional endothelium [6]. When normal endothelial function is disrupted, several vasoactive and thrombotic/antithrombotic substances are no longer balanced. The following abbreviations are employed in the figure: arrow, promotion; ---, inhibition; ET, endothelin; FDP, fibrin degrading products; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteinase; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PGI2, prostaglandin; SMC, smooth muscle cell; TF, tissue factor; tPA, tissue plasminogen activator; TXA2, thromboxane A2; VEGF, vascular endothelial growth factor.
Figure 1.3: The role of the endothelium in plaque development. Endothelial dysfunction and inflammation lead to the initiation and progression of atherosclerotic lesions. Reproduced from [6].

Sudden rupture of a vulnerable plaque has been observed to occur spontaneously without obvious external triggers. However, plaque rupture may also follow a specific event, such as extreme physical activity, severe emotional distress, sexual activity, exposure to illicit drugs (coca, amphetamines), and exposure to cold or acute infection [19]. Regardless of cause, plaque rupture often leads to thrombosis with the clinical manifestations of an acute coronary syndrome (ACS); however, rupture may also occur without clinical consequences, which is often referred to as silent plaque rupture. Autopsy data show that 9% of “normal” healthy persons have asymptomatic ruptured plaques in their coronary arteries, increasing to 22% in persons with hypertension or diabetes [20]. Furthermore, in approximately 40 to 80% of cases of ACS, multiple
plaque ruptures have been demonstrated in arteries remote from the acute rupture site [21]. The thrombotic response to a plaque rupture is regulated by the thrombogenicity of the exposed plaque constituents, the local hemorrheology, shear-induced platelet activation, and by systemic thrombogenicity and fibrinolytic activity [22].

1.4 Current Detection Methods for Vulnerable Plaque

There are many imaging techniques currently in clinical use that have the potential to detect vulnerable plaques. Coronary angiography is an X-ray examination of the blood vessels or chambers of the heart and has been the gold standard to assess the severity of obstructive luminal narrowing. Coronary angiography has the ability to assess the lumen boundaries, but it cannot assess the plaque burden and its delineation and components. The predictive power of myocardial infarction occurrence is quite low since 70% of acute coronary occlusions are in areas that were previously angiographically normal, and only a minority occur where there was severe stenosis [23]. Coronary angiograms also often fail to identify the culprit lesion of non-transmural myocardial infarction. The biggest drawback to coronary angiography is that patients with silent non-obstructive coronary atherosclerosis can harbor vulnerable plaques that cannot be detected by angiograms.

Intracoronary angioscopy offers direct visualization of the plaque surface and intraluminal structures via a miniature endoscope, called an angioscope, with high resolution optics coupled to a small video camera. It allows assessment of the color of the plaque and thrombus with higher sensitivity compared to angiography [24]. As mentioned previously, yellow plaques seem to have an increased instability due to the
presence of a very thin cap covering a lipid pool, and these plaques can be imaged with angioscopy but not with angiography. The major limitations of angioscopy are obvious: angioscopy is difficult to perform, is invasive, and only a limited part of the vascular tree can be investigated. Furthermore, to enable clear visualization of the vessel wall, the vessel must be occluded and the remaining blood flushed away with saline, which may potentially induce regions of myocardial ischemia.

Intravascular ultrasound (IVUS) provides real-time high-resolution images of the vessel wall and lumen using miniaturized high-frequency (20 – 30 MHz) transducer assemblies inserted into catheters [25]. Features of the vessel can be detected based on the echogenicity and the thickness of the material. IVUS also provides some information about the composition of coronary plaques. In IVUS images, calcification is characterized by a bright echo signal with distal shadows that hide plaque components and deeper vessel structures. Lipid depositions in IVUS images are described as echolucent zones, but the interpretation of these areas is difficult. The biggest potential for IVUS in detecting vulnerable plaque is that IVUS is able to assess vascular remodeling, which is associated with plaques that have the highest probability of spontaneous rupture [26].

A thermographic approach to plaque detection is based on the hypothesis that a temperature rise can be measured at the surface of a plaque, which is caused by the inflammation process of atherosclerosis. Stefanadis et al. have performed studies in humans to test this hypothesis [27]. Patients with stable angina, unstable angina, and acute myocardial infarction were studied. The thermistor (temperature-dependent
resistor) of the thermography catheter was driven against the vessel wall by the force of blood flow, without the help of a mechanical device like a balloon. Temperature was constant within the arteries of the control subjects, while most atherosclerotic plaques showed higher temperatures compared with healthy vessel walls. Temperature differences between atherosclerotic plaque and healthy vessel walls increased progressively from patients with stable angina to patients with acute myocardial infarction with a maximum difference of 1.5 °C. However, these results have not yet been confirmed prospectively in other medical centers, and the influence of parameters such as coronary blood flow and catheter design needs further study.

Optical coherence tomography (OCT) is a technique that measures the intensity of back-reflected light in a similar way as IVUS measures acoustic waves. The result is images with ultra-high resolution [28]. OCT is an invasive technique that uses a Michelson interferometer to split light into two signals. One signal is sent into the tissue and the other is sent to a reference arm with a mirror. The intensity of the interfering signals at a certain mirror position represents backscattering at a corresponding depth. Resolution ranging from 4 – 20 μm can be achieved with a penetration depth up to 2 mm, and images can be acquired real-time. A lipid pool generates decreased signal areas, and a fibrous plaque produces a homogeneous signal-rich lesion. However, the low penetration depth hinders studying large vessels, and the light absorbance by blood must still be overcome by saline infusion or balloon occlusion as is required with angioscopy.

Near-infrared (NIR) spectroscopy provides information of the chemical components of the coronary vessel wall. Molecular vibrational transitions measured in
the NIR region (750 - 2500 nm) give qualitative and quantitative results on plaque composition. A differentiation between vulnerable and non-vulnerable carotid plaques was achieved ex vivo [29]. The potential problems with this technique in vivo involve acquisition time, blood scattering, and influences of temperature and pH, which will need to solved for this technique to become prognostic.

High-resolution magnetic resonance imaging (MRI) is a non-invasive modality that is often used to characterize atherosclerotic plaques. Imaging of coronary arteries with MRI is more difficult than imaging carotid plaques since cardiac and respiratory motion, the small plaque size, and the location of the coronary arteries can cause acquisition problems. Currently, small plaque structures like fibrous caps cannot be assessed using current MRI techniques; thinner slices and higher in-plane resolution are needed to better delineate coronary plaques [30].

1.5 Targeting Vulnerable Plaque

It is clear from the previous discussion that the major limiting factor of all current detection methods is the lack of specific targeting capability. Furthermore, many of the current imaging modalities are invasive, which is not ideal for the patient. Thus, the delivery of image contrast particles and/or therapeutics to sites of disease is a major goal to enhance clinical benefit. Many targeting strategies exist, including methods that exploit the “enhanced permeability and retention” effect in tumors [31]; the preferential partition of molecules and particles into specific tissues based on their sizes, charges, or surface chemistry; or the affinity of biological molecules attached to a nanoparticle/nanodevice for counter-receptors on the cells or tissues of interest.
Targeting based on biological affinity reagents attached to the surfaces of image contrast or therapeutic nanodevices is perhaps the most direct approach to solving the targeting problem.

The incorporation of biological macromolecules is unavoidable for many nanodevices that offer imaging contrast and therapeutic activity. This is primarily due to the fact that any imaging or therapy will occur in a biological system (e.g., human beings). This fact has led to the use of the word “nanobiotechnology” to indicate the dependent nature of the nano and biological components of the devices. Proteins such as antibodies have the capability to recognize specific molecular markers but are typically much more expensive than small molecule therapeutics, and developing precise nanostructures containing proteins will be even more costly. Another important consideration is that nanotherapeutics may carry larger risks than those associated with today’s conventional therapies. Thus, these new and perhaps expensive nanobiological devices will be most likely accepted into everyday medical use if the following two criteria are met: (a) the disease is accessible to intervention at the nanoscale, and (b) existing imaging and therapeutic techniques have well-accepted limitations. The identification and treatment of the vulnerable plaque clearly meets these two criteria, and thus justifies the design of novel nanobiological devices.

1.5.1 Current Targeting Capabilities via Nanoparticles

Nano-sized particles in the range of 5 – 100 nm in diameter have been used as contrast-enhancing agents for MRI [32]. The first generation of MR contrast agents were primarily systemic agents that circulated throughout the entire vasculature. Targeting
contrast-enhancing nanoparticles to disease sites can reduce both overall cost and heavy metal toxicity associated with the commonly used agents by drastically lowering the dose required to obtain differential imaging capability.

The general scheme of a targeted nanoparticle for image-enhancing contrast is shown below in Figure 1.4 [33]. The schematic shows several features of nanodevices that are common to many current targeting strategies. The molecule of interest, T, is some specific surface marker that indicates a disease state, for example, some molecular feature that distinguishes vulnerable from stable plaque. This molecule is targeted by a specific ligand, L, which could be an antibody (or other protein) or small peptide. The ligand is conjugated to a carrier particle, C, which could be cells or polymers. Finally, the signal molecule, S, provides the contrast-enhancement under the imaging modality of interest. For MRI this signal molecule is often ultra-small superparamagnetic iron oxide (USPIO) particles or a gadolinium-containing contrast agent. The signal detector in this case would be the MR signal detected during image acquisition.
Figure 1.4: A generic model for targeted contrast-enhancing nanoparticles. Several features of nanodevices are common to many current targeting strategies. The molecule of interest, $T$, is some specific surface marker indicating a disease state and is targeted by a specific ligand, $L$, which could be an antibody or small peptide. The ligand is conjugated to a carrier particle, $C$. Finally, the signal molecule, $S$, provides the contrast-enhancement under the imaging modality of interest. Reproduced from [33].
One such combination of target molecule and ligand regarding atherosclerosis is phosphatidylserine (PS) and annexin V. PS is a phospholipid that is asymmetrically expressed on the cellular membrane such that most of the PS is intracellular under normal conditions. As shown in Figure 1.5, during apoptosis (programmed cell death), the asymmetry of lipid distribution of the membrane breaks down, and PS becomes pronounced on the outer surface of the cells, accessible to the flowing blood. Annexin V is a well-known protein that binds PS and has been used as a targeting agent for MR image contrast [34]. The important hypothesis for the use of the PS/annexin V system is that there is a high incidence of apoptosis in regions of atherosclerosis. Thus, PS is thought to be overexpressed in diseased regions of the vasculature and annexin V can be used to target these regions.
Figure 1.5: **Schematic representation of the loss of membrane asymmetry during apoptosis.** Phosphatidylserine (PS, indicated by red spheres) is asymmetrically distributed in the cell membrane such that under normal conditions it is facing the cytosol. During apoptosis, the integrity of the membrane is lost, and PS becomes exposed on the outer membrane leaflet. Annexin V can bind PS with high affinity in the presence of calcium ions. Image reproduced from [http://www.bdbiosciences.com](http://www.bdbiosciences.com).

Annexin V has been attached to USPIOs consisting of an iron oxide core and dextran polysaccharide coating (Miltenyi Biotec, Gladbach, Germany). In vivo demonstration of MRI contrast has been shown (Bryan Smith, Ph.D. dissertation, OSU) in a rabbit model of atherosclerosis that will be discussed later. It is yet to be conclusively determined that the annexin V binds to PS in regions experiencing increased apoptosis. Furthermore, the possibility that annexin V is not the “best” targeting agent for atherosclerotic plaque provides the impetus for the search of de novo compounds.

**1.5.2 Novel Targeting Approach**

Several candidate markers for atherosclerotic plaque have been recognized (e.g., annexin V, LOX-1, VCAM-1), but as of yet none has shown to conclusively mark the
presence of vulnerable plaque. An ideal marker for vulnerable plaque would be a biomolecular marker that is present only on vulnerable plaque and is absent on stable plaque or healthy tissue. Finding such a marker would open up a wide variety of diagnostic and therapeutic capabilities that cannot be realized currently. It is probably an obvious fact that finding a vulnerable plaque-specific marker is a challenging task, because it has not yet been accomplished, but there are techniques that allow the so-called “vascular address” system to be probed for specific markers [35]. The principal method for molecular probing is phage-displayed random peptide libraries.

Phage display describes a selection technique in which a peptide or protein is expressed as a fusion with a coat protein of a bacteriophage, resulting in display of the fused peptide or protein on the surface of the virion. Large numbers of virions occupy only a small volume (10^{10} – 10^{12} particles/mL), so it is possible to construct vastly diverse libraries using recombinant DNA technology, wherein each phage displays a unique random peptide. DNA segments that encode the random peptides are inserted into the phage genome and become expressed as the peptides that are fused to the protein coat of the phage. Thus, phage display creates a physical linkage between the library of random peptide sequences to the DNA encoding each sequence. Because DNA can be rapidly sequenced, the peptides displayed on selected phage molecules are easily identified.

Figure 1.6 shows a schematic of a lambdoid bacteriophage, which is provided only to illustrate basic properties of phage in general. The DNA that encodes the peptide resides in the head of the bacteriophage. Billions of unique displayed peptides can be
created by randomly inserting (cloning) DNA sequences into the phage. Figure 1.7 shows a diagram of a filamentous phage with inserted DNA that expresses a fusion peptide, which is the structure of the phage seen in the phage display libraries. The interested reader is referred to [36] for a review of phage display and its applications.

Figure 1.6: **Schematic of a lambdoid bacteriophage.** Bacteriophage are intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery, *i.e.*, they are viruses that infect bacteria. Note that this particular form of phage is not used in this work.
Figure 1.7: **DNA inserted into the filamentous phage genome displays a peptide on the surface of the phage.** The DNA encodes the displayed random peptides, thus creating a physical link between phenotype and genotype. Reproduced from [http://cmbi.bjmu.edu.cn/](http://cmbi.bjmu.edu.cn/).

An in vitro selection process called “panning” allows rapid identification of peptide ligands for a variety of target molecules. In its simplest form, panning is carried out by incubating a library of phage-displayed peptides with a plate coated with the target, washing away the unbound phage, and eluting the specifically-bound phage as shown in Figure 1.8. The eluted phage are then amplified and taken through additional binding/amplification cycles to enrich the pool in favor of binding sequences. After three or four rounds, individual clones are characterized by DNA sequencing [37].
Figure 1.8: **Basic “panning” process of in vitro phage display.** The cycle is repeated three to four times before the phage DNA is sequenced to determine the displayed peptide.

Figure 1.9: **In vivo “biopanning” process of affinity maturation.** The cycle is similar to that in Figure 1.8; however, phage are circulated in vivo in an animal model rather than incubated on a plate containing the target.
In a similar process to the in vitro panning shown in Figure 1.8, phage display can also be used to identify peptides that bind to specific targets in vivo. This technique is termed "biopanning" and is represented schematically in Figure 1.9. The method involves injecting a phage display library into an animal model, allowing the phage to circulate for a prescribed amount of time, sacrificing the animal, retrieving the bound (and possibly internalized) phage from the tissues or organs of interest, amplifying the recovered phage by infecting bacteria, and reintroducing the recovered phage pool into a new animal. This cycle is repeated three to four times to select the phage that display the peptides with the highest affinity for the target. In this case, a rabbit model of atherosclerosis is used (described later), and the tissue of interest is the aorta.

The major benefit of this targeting strategy is that no a priori knowledge of the target is required to identify a potential ligand. Rather, in a somewhat brute-force method, the target is probed with a vastly diverse pool of prospective ligands, and the peptides with greatest affinity for the target are selected after multiple rounds of panning. A major drawback of this approach aside from technical difficulties of panning is that peptides themselves often have lower affinity for their targets than proteins that bind the same site. This is due to the fact that the 3-D structure of proteins precisely positions a binding sequence in space to match its ligand; peptides, having much shorter primary amino acid sequences, may not be able to optimize the 3-D orientation of the sequence for strong binding. This limitation can potentially be overcome by increasing the valency of the targeting agent. For example, two or more peptides that recognize different
epitopes of the same target can act in concert to increase the effective binding constant of the target-ligand complex.

1.6 Summary and Statement of Goals

There is a clear and urgent need for the development of a screening diagnostic to determine patients harboring vulnerable atherosclerotic plaque. A novel method of targeted image contrast has been described that depends on biological nanodevices as the foundation. The use of phage displayed peptide libraries provides an opportunity to identify novel biological targeting agents that bind plaque. Such targeting agents can be conjugated to image-enhancing iron oxide nanoparticles in order to screen at risk patients using current medical imaging modalities (e.g., MRI). Furthermore, the development of a targeted nanodevice to plaque can serve as a platform for future nanotherapeutics.

The specific goals of this work are as follows:

- Identify de novo targeting agents (peptides) that bind atheromatous plaque in the Watanabe (diseased) animal model using phage display.

- Demonstrate the in vivo binding of selected peptides to plaque.

- Demonstrate specificity of targeting to plaque by testing peptides on a control rabbit model, the New Zealand white rabbit.

- Determine if selected peptides can bind to lipid-loaded human aortic endothelial cells (HAECs), which would demonstrate the applicability of the animal model to humans.

- Determine the location of targeted peptides on the stained tissue.

- Isolate the molecular cognate(s) of selected peptides from the animal model.
By completing the above objectives, several important steps toward developing a diagnostic and therapeutic cardiovascular nanodevice will be made. Identifying novel peptides that bind plaque will create a suite of targeting agents that can be used as ligands on a contrast-enhancing nanoparticle or to deliver a protein therapeutic to a site of vascular disease. It is possible that these novel targeting agents will provide AHA specificity to different grades of plaque (see Figure 1.1). Furthermore, these targeting agents may provide biomolecular information regarding susceptibility of plaque to rupture, i.e., the vulnerability of the plaque. Such information would be invaluable to a clinician when identifying patients at risk for ACS and when attempting to develop treatment strategies.
CHAPTER 2
MATERIALS AND METHODS

2.1 Phage Display Libraries

The potential candidates for peptides that target plaque were all discovered using two commercially available phage display peptide library kits (New England Biolabs, MA). The Ph.D.-12 kit consists of 12-mer amino acid sequences and contains approximately $1.9 \times 10^9$ independent clones. The second kit is the Ph.D.-C7C, which is comprised of $1.2 \times 10^9$ 7-mer sequences that are flanked on both sides by cysteine residues, thus producing 9-mers of the form Cys-(7-mer)-Cys. Under nonreducing conditions the cysteines spontaneously form a disulfide cross-link, which results in phage display of cyclized peptides, in contrast to the linear 12-mers.

2.2 Experimental Animal Model of Atherosclerosis

The animal model used in this work is the Watanabe heritable hyperlipidemic (WHHL) rabbit. The WHHL model is genetically predisposed toward the formation of atherosclerotic plaques [38], and the animals were purchased from either Covance (Princeton, NJ) or Brown Family Enterprises/Gemini Research (Odenville, AL). The WHHL rabbit develops fairly occlusive plaque in the aorta increasingly with age. The disease seen in this strain is quite similar to the human condition of familial
hyperlipidemia, as demonstrated by the fact that the lipoprotein type found in the vascular
tissues is nearly identical. The WHHL rabbit develops not only atherosclerosis, but also
xanthomas (fat deposits under the surface of the skin), fatty infiltration of organs, and
elevated lipids on a normal diet. Usually, severe coronary artery disease occurs by age
two in these animals. While this animal model is generally accepted, there are some important
differences between the disease progression in humans and WHHL rabbits. Primarily, the animals’ cholesterol metabolism is fundamentally different, because
rabbits are herbivores. In addition to this, the primary location of atherosclerosis in man
is the abdominal aorta, but develops in the aortic arch and thoracic aorta in the WHHL
model. Nonetheless, the WHHL rabbit model provides an easily accessible source of
plaque-laden vasculature that can be used to isolate targeting agents for atherosclerotic
plaque.

Recently, a spontaneous myocardial infarction (MI) version of the model, the
WHHL-MI, has been developed [39], and is used in some of the experiments as well
(Masahashi Shiomi, Kobe, Japan). WHHL-MI rabbits experience spontaneous lethal
vascular events and have plaques with similar morphology to the vulnerable plaques seen
in humans. Furthermore, the plaques in the WHHL-MI model often exhibit evidence of
prior rupture, which is the case in humans as well [40]. In spite of this improvement with
respect to modeling the human disease, the WHHL-MI is still not a perfect model. It has
been shown that in most cases, vulnerable plaque rupture is not the primary cause of the
fatal coronary events experienced by most of the WHHL-MI rabbits; additional triggering
factors are required to promote plaque rupture and thrombogenesis. However, the animal models are a good compromise between disease progression, cost, and accessibility.

The control animal for the WHHL/MI rabbit models is the New Zealand white (NZW) rabbit. This animal rarely shows evidence of large lesions, and will typically only develop plaque when fed a high cholesterol diet. Like the WHHL animals, the NZW rabbits are supplied by Covance (Princeton, NJ). All animal studies were approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee and performed in compliance with accepted standards of laboratory animal care.

2.3 Isolation of Peptides that Bind Plaque: Biopanning

The initial biopanning experiments were performed by Mr. John Shapiro, and the following paragraph follows from his work. To isolate peptides that bind specifically to the diseased intima in the atherosclerotic lesions of the WHHL rabbits, both the Ph.D.-12 and -C7C libraries were administered simultaneously in the ear vein of the animal. The phage were suspended in a small volume of Hanks buffered salt solution and circulated for 30 minutes in the anesthetized animal. Following the incubation with the phage libraries, the animal was perfused through the left ventricle with 200 – 300 mL of Hanks buffered salt solution to wash away nonspecifically bound phage in the aorta. The aorta was then removed, and the bound phage were eluted according to the protocol provided by New England Biolabs. The eluted phage were amplified in the recommended host bacteria, purified, and injected into another WHHL rabbit. This process was repeated in four rabbits, and the sequences of 400 isolated peptides were determined from the DNA sequences of the individual phage clones.
2.4 Isolation of Peptides that Bind Lipid-Loaded HAECs: Panning

Human aortic endothelial cells were purchased from Cambrex (CC2535) and cultured in the associated media according to the recommendations of the company. Cells were grown to confluency after which time acetylated low-density lipoprotein from a human source (Invitrogen, L35354) was added to the media at a concentration of 15 μg/mL. Cells were fed lipid-loaded media every three days for a total of three feedings. Panning with the phage libraries was conducted as previously described and was concluded after three rounds.

2.5 Selection of Targeting Peptides

The isolated peptides from the in vivo biopanning on WHHL rabbits and the in vitro panning on lipid-loaded HAECs were analyzed to determine if any homology was present throughout the different rounds. Several sequences were selected based on this criterion that show consensus sequences 3 – 5 amino acids in length. Another criterion for selection was if individual peptides were identified repeatedly during the various rounds of biopanning on WHHL rabbits. BLAST searches\(^1\) were also performed on all the isolated peptides to identify peptide sequences that are present in proteins associated with atherosclerosis. Peptides with 6/7 or 9/12 conserved sequences, depending on the length of the amino acid sequence, were considered for potential targeting agents. More than 40 individual peptide sequences were selected and synthesized by GenScript Corporation (Piscataway, NJ) with >85% purity for all peptides. The peptides were

\(^1\) http://www.ncbi.nih.gov/BLAST/
synthesized with either an N-terminal biotin or a C-terminal FLAG sequence, DYKDDDDK, for the detection assays.

2.6 Description of Peptide Assays

Several experimental methods were employed to validate the binding capabilities of the synthesized peptides. The following discussions describe the assays.

2.6.1 Lipid-Loaded HAECs

Human aortic endothelial cells (HAECs) were loaded with acetylated low-density lipoprotein as described in Section 2.4. Peptide binding assays were performed 24 hours after the third lipid-loading of the cells. Control cells grown in the standard media without added lipid were cultured for the same amount of time as the lipid-loaded HAECs.

The general procedure for the peptide binding assays was conducted as follows (see Figure 2.1). Variations on this method will be discussed as well. Both the lipid- and non-lipid-loaded cells were imaged prior to the assays to verify morphological differences between the two conditions of the cells. The media was aspirated and cells were washed 3X with cold wash buffer (PBS w/ Ca$^{2+}$, Mg$^{2+}$ with 2% FBS), followed by a 5 minute incubation on ice to minimize internalization of peptides. Cold blocking buffer (PBS w/ 1% BSA) was added to the cells for 15 minutes to prevent nonspecific binding. Peptides were then added to the cells in cold blocking buffer at 1, 5, or 100 µg/mL and incubated for 1 hour on ice. Following incubation, cells were washed 3X, and either streptavidin horseradish peroxidase (SA-HRP) or anti-FLAG horseradish peroxidase (anti-FLAG-HRP) was added to the cells in cold blocking buffer at the recommended
concentrations provided by the manufacturers. Anti-FLAG monoclonal m2 antibody-peroxidase conjugate was purchased from Sigma (A8592), as well as SA-HRP (S2438). After a 1 hour incubation, cells were washed 5X and were incubated with diaminobenzidine (DAB, Sigma, D5905) for 30 minutes (1 DAB tablet, 15 mL wash buffer, 12 μL 30% H₂O₂, followed by filtering), which results in a brown colored precipitate in the presence of HRP. Following the incubation with DAB, cells were washed 3X and fixed with 10% buffered formalin for 10 minutes. Following a final wash to remove the formalin, cells were imaged to determine if brown staining was present. SA-HRP and anti-FLAG-HRP were added directly to cells that had not been incubated with peptide as a control to determine if the presence of brown truly indicated positive peptide binding.

**Figure 2.1:** Schematic of HAEC peptide binding assay. Lipid- and non-lipid-loaded human aortic endothelial cells were assayed to determine if the synthesized potential targeting peptides could bind human cells.
Several variations on this general assay were performed to find an optimal assay system. As mentioned previously, peptide concentrations ranged from 1 to 100 µg/mL throughout the experiments. In one variation of the assay, peptides were added directly to the media, bypassing the washing and blocking steps. In this case the cells were incubated at 37 °C, 5% CO₂ for 1 – 2 hours. This technique offered a greater chance that the peptides could be internalized by the cells compared to the cold version described above, so it was necessary to permeabilize the cells before adding SA-HRP or anti-FLAG-HRP. This was performed by adding either a 0.5% Triton-X solution for 10 minutes followed by a 20 minute incubation with 0.03% hydrogen peroxide, or by first fixing the cells with 4% paraformaldehyde for 15 minutes followed by permeabilization with cold methanol for 10 minutes.

The composition of the blocking buffer was also varied throughout the experiments. Initially, the blocking buffer contained 2% FBS, 1% BSA, 0.1% cold fish gelatin, and 0.05% sodium azide. Other compositions were 2% BSA, 1% BSA, and 0.5% BSA. The effect of counterstaining with hemotoxylin was also tested in some experiments either before or after fixing the cells to observe if brown staining was easier to detect on stained cells. The controls for this experiment were comparing the binding of each peptide to lipid- and non-lipid-loaded cells, and two wells of cells were incubated with only blocking buffer (no peptide) to test for binding of the SA-HRP and anti-FLAG-HRP to the cells.
2.6.2 Resected WHHL and NZW Rabbit Tissue

Several experiments were performed to determine if the synthesized peptides could bind to the intima of the WHHL (diseased) rabbits but not to the NZW (control) rabbits. The following procedure is shown schematically in Figure 2.2. The animals were anesthetized, injected with 0.7 – 1.0 mL heparin, and perfused with 200 – 300 mL Hanks buffered salt solution containing 1% BSA. A segment of the aorta comprising the aortic arch to the bifurcation of the aorta in the lower abdomen was removed and placed in a buffer solution of Hanks w/ 1% BSA and 1% protease inhibitor cocktail (Sigma, P8340). The aorta segments were opened longitudinally and sliced into individual pieces approximately 1 – 2 mm² each. These pieces were then placed into tubes containing individual peptides at a concentration of 5 µg/mL in the same buffer solution described above.

The tissue sections were incubated with individual peptides for 45 – 60 minutes at room temperature. Samples were washed 3X with PBS followed by incubation with SA-HRP or anti-FLAG-HRP in the same buffer for 45 – 60 minutes at room temperature. After another 3X washing, the samples were incubated with DAB for 30 minutes in the same buffer. After a final round of washing, tissue samples were imaged with a dissecting microscope and analyzed for the presence of brown staining on the intimal surface. Tissue sections that showed significant staining were fixed with 10% buffered formalin and sectioned for further analysis to determine the location of the staining (e.g., endothelium, smooth muscle). Some tissue sections received no peptide incubation; rather they were exposed only to SA-HRP or anti-FLAG-HRP as a control.
Figure 2.2: Schematic of resected tissue peptide binding assay. Peptides were tested on both WHHL and NZW tissues to test for binding to aortic intima.

2.6.3 Direct Staining on Frozen WHHL and NZW Rabbit Tissue

Some of the resected WHHL and NZW aortic arch tissues were frozen and sectioned into 4 μm slices. Binding assays were performed on these frozen sections in a similar manner to that described in Section 2.6.2. Theo Nicholson helped conduct several of these experiments. Sections were thawed and incubated with blocking buffer (Hanks w/ 1% BSA) for 30 minutes. Individual peptides were added to the slides in blocking buffer at a concentration of 1 μg/mL. After 45 minutes of incubation, the slides were washed and either SA-HRP or anti-FLAG-HRP, depending on the modification of the peptides, was added at the recommended concentration and allowed to incubate for 1 hour. The slides were washed again, and the DAB substrate was added and incubated for 30 minutes while looking for brown color development. After washing away the DAB, the tissue samples were fixed with 10% buffered formalin for 10 minutes followed by
counterstaining with hemotoxylin. The slides were then mounted with permount and glass coverslips for imaging. The standard SA-HRP and anti-FLAG-HRP controls were performed for these experiments as well.

2.6.4 ApoE Knockout Mice

Recently, some initial experiments have been performed by John Shapiro on another animal model of atherosclerosis: the ApoE knockout mouse. The ApoE glycoprotein is involved with cholesterol transport activity among cells, and the knockout animal model exhibits five times the normal serum plasma cholesterol level as the control model and develops spontaneous atherosclerotic lesions that are similar in morphology to the human disease [41]. In the peptide binding assays, individual peptides are injected into an anesthetized mouse and allowed to circulate in vivo for 1 hour. Note that this is different from the WHHL/NZW binding assays where the aorta is first resected and then incubated with peptide. The animal is then fixed via cardiac perfusion with 4% formaldehyde, and the aorta is removed to test for binding of the peptide. The detection assay is the same assay described in Section 2.6.2: either SA-HRP or anti-FLAG-HRP is added to the solution containing the resected tissue, followed by the addition of the DAB substrate, after which the tissue samples are sectioned and imaged.

2.7 Pull-down Experiment

An initial attempt was made to isolate the protein to which the targeting peptides bind in vivo using a pull-down type experiment. The technique utilized to “pull down” the protein of interest is a magnetic separation. Iron oxide nanoparticles coated with streptavidin (Miltenyi Biotech, Germany) are washed to remove the azide from the buffer
solution and are incubated with biotinylated peptide 9 to create a targeting agent for the protein that can be isolated via magnetic separation (see Figure 2.3). The experiment begins by resecting a portion of WHHL aorta and homogenizing the tissue sample in a glass dounce (Kontes, 885300-0002). The Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce Biotechnology, 89826) is used to isolate the hydrophobic membrane proteins where it is assumed the protein to which the targeting peptide binds may be found. The SA-coated iron oxide nanoparticles conjugated to the biotinylated peptide are added to the isolated membrane protein solution. The peptide concentration is approximately 15 μg/mL. The mixture is incubated for 2 hours at 4°C and is then placed in a magnetic separator and left overnight at 4°C to separate the bound protein from the bulk proteins.

After the initial overnight separation, the supernatant is collected and the sample is washed with PBS containing protease inhibitors (Sigma, P8340). The sample is incubated in the magnetic separator at 4°C for 3 – 4 hours followed by a second wash step and another 3 – 4 hour separation. After the final separation the supernatant is poured off and the particles are centrifuged at 1000 rpm for 2 minutes in order to resuspend the particles in the remaining buffer. This avoids diluting the protein concentration by adding more buffer. The sample is then prepared for gel electrophoresis.

The NuPAGE Novex gel system from Invitrogen is used for protein separation. The 4-12% Bis-Tris gel (NP0321BOX) is combined with the MES SDS running buffer (NP0002) to create a system with a large protein size separation range (2.5 – 200 kDa).
The sample buffer is the NuPAGE LDS sample buffer (NP0007), and all samples are prepared with sample reducing agent (NP0004) and run with antioxidant (NP0005) in the running buffer to avoid reoxidation during the electrophoresis. Samples are prepared according to the NuPAGE system manual, and the gel is run using the XCell SureLock (EI0001) mini-cell system. A protein standard (SeeBlue Plus2, LC5925) is run on the gel for size estimation. After the electrophoresis the gel is stained with Coomassie Blue (SimplyBlue SafeStain, LC6060) to visualize protein bands. Bands of interest are then excised for mass spectrometry analysis.
Figure 2.3: Schematic of pull-down experiment. This experiment is an attempt to isolate the protein to which a potential plaque-targeting peptide binds. The membrane proteins are isolated from WHHL rabbit aorta using the Mem-PER extraction kit from Pierce and are incubated with SA-coated iron oxide nanoparticles that are conjugated to biotinylated peptide. The bound protein is separated magnetically, run on SDS-PAGE gel electrophoresis, and analyzed with mass spectroscopy.
CHAPTER 3
RESULTS

3.1 Biopanning Results

In vivo biopanning was performed on the WHHL rabbit animal model as described in Section 2.3 using both the 12-mer linear peptide and 7-mer disulfide bond constrained peptide phage display libraries. Panning was also performed on lipid-loaded HAECs as described in Section 2.4 using both libraries as well. From the panning procedures over 200 novel peptides were discovered de novo that are potential targeting agents for atherosclerotic plaque. A complete listing of all the isolated sequences is given in Appendix A (Tables A.1 and A.2). Forty five of these candidates were selected to be synthesized for further binding assays based on specific selection criteria. These peptides are shown in Table 3.1. The peptides were modified by either N-terminal biotinylation or C-terminal FLAG sequence, DYKDDDDK. The biotinlyated peptides are assayed for binding using SA-HRP, while the FLAG sequence is detected by anti-FLAG-HRP (see Section 2.6).
<table>
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<th>Sequence</th>
<th>N-terminal Biotinylation</th>
<th>C-terminal FLAG (DYKDDDDK)</th>
<th>Peptide Number</th>
<th>Sequence</th>
<th>N-terminal Biotinylation</th>
<th>C-terminal FLAG (DYKDDDDK)</th>
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Table 3.1: **Peptides selected for further binding assays.** The 45 peptides listed above were selected from the biopanning on WHHL rabbits and the panning on lipid-loaded HAECs. The peptides were modified by either N-terminal biotinylation or C-terminal FLAG sequence, DYKDDDDK, for the detection assays. The specific modification for each peptide is indicated by the shaded box.

A major factor in selecting a peptide was whether it showed homology to other peptides isolated from individual rounds of panning. For example, the peptide C(KQSFKKS)C was selected based on its homology to the sequences KQSPPSM and
KQSQWHS, where the flanking cysteine residues are shown for only the first peptide for simplicity; the reader should be aware that 7-mers described in this discussion are all flanked by cysteine residues. The fact that certain peptides showed distinct homology to others isolated from the panning experiments did not necessarily develop a criterion for which of the homologous peptides to select. Therefore, there existed some arbitrary selection based on the homologous criterion. Another major selection criterion was if a particular peptide sequence appeared throughout multiple rounds of the panning experiments. An example of this is the 12-mer, TPLEVHPESLPW, which appeared in two rounds of biopanning. Furthermore, this sequence shows some homology to another isolated 12-mer, TPLTPNGLTRSG, which increased the potential for this peptide to be a good targeting agent. The final selection criterion was the use of a BLAST search (see Section 2.5). Some peptides showed homology to known proteins associated with atherosclerosis or inflammatory processes in general. An example of this homology is the 12-mer, TPLTPNGLTRSG, which was mentioned above as being homologous to a 12-mer that appeared in two rounds of biopanning. This peptide shows good homology to a LDL receptor related protein (conserved sequence: LTPNGLT), further indicating the potential for this peptide to target plaque.

Three peptides were synthesized that were not selected from the biopanning on WHHL rabbits or the panning on lipid-loaded HAECs. These peptides have the following sequences: CNHRYMQMC (peptide 11), CAPGPSKSC (peptide 12), and SIGYPLP (peptide 13). The first two peptides were selected based on the results of Liu et al., which describe the use of a cysteine-constrained phage displayed peptide library to
probe the surface of atherosclerotic lesions [42]. The first peptide, CNHRYMQMC, was shown to bind to the atherosclerotic lesion endothelium of ApoE knockout mice in vivo, while the second peptide, CAPGSKSC, bound to both ApoE knockout mice in vivo and also human atherosclerotic lesions ex vivo. The third peptide, SIGYPLP, was found to bind human umbilical vein endothelial cells by Nicklin et al. [43]. It is also important to note that peptides 43, 44, and 45 in Table 3.1 are randomly scrambled variants of peptides 9, 3, and 4. The utility of these peptides will be discussed in Section 3.2.2.

3.2 Peptide Binding Assay Results

The assays described in Section 2.6 were conducted in an attempt to demonstrate the binding capabilities of the peptides isolated from phage display. The results of these assays are described in the following sections.

3.2.1 Lipid-Loaded HAECs Peptide Binding Results

As described in Section 2.6.1, human aortic endothelial cells (HAECs) were loaded with media containing LDL, which mimics an early process in atherosclerotic plaque development. HAECs without added LDL were simultaneously cultured to provide a control for the lipid-loaded cells. To demonstrate uptake of lipid by the HAECs, images of both cell conditions were acquired, and the results are displayed in Figure 3.1. The image shown in (a) shows the HAECs after one week of lipid-loading, while the image shown in (b) shows the control cells without added lipid. The arrows in (a) indicate regions of accumulated lipid. Note that no such morphological areas are seen in (b). Both images are 400X magnification. The difference in the photographic quality of the images is due solely to the software used to acquire the images (SPOT).
Figure 3.1: Demonstration of successful lipid loading of HAECs. (a) Lipid-loaded HAECs after one week of growing in media containing 15 μg/mL acetylated LDL. The arrows indicate distinct accumulation of lipid. (b) Control cells without added lipid.

The binding assay on the lipid-loaded HAEC system (Section 2.6.1) was performed for peptides 1 – 33, excluding peptide 8 (see Table 3.1 for peptide listing). Peptides 8 and 34 – 45 were not tested for binding, because they were not synthesized at the time the experiments were conducted. The experiments indicate that no positive binding was observed for any of the peptides that were assayed on the lipid-loaded HAECs. A representative sample of results is shown in Figures 3.2 and 3.3. Both images show no brown staining, which indicates a negative result for binding. Figure 3.4 shows images from the SA-HRP and anti-FLAG-HRP controls. The control experiments show negative results for binding, which indicates that the detection antibody or protein does not bind to HAECs itself.
Figure 3.2: **Result of lipid-loaded HAEC assay for peptide 17.** Absence of brown staining indicates negative result for binding. Image is 100X magnification.

Figure 3.3: **Result of lipid-loaded HAEC assay for peptide 23.** Absence of brown staining indicates negative result for binding. Image is 100X magnification.
Figure 3.4: **Result of lipid-loaded HAEC assay for anti-FLAG and SA controls.** Absence of brown staining indicates negative result for binding, which is a positive result for the case of the control experiments. (a) Result for anti-FLAG control, 200X. (b) Result for SA control, 200X.

### 3.2.2 Resected Aortic Tissue Binding Results

All peptides in Table 3.1 were assayed for binding to both WHHL and NZW rabbit aortic tissue as described in Section 2.6.2. The most generalized statement about the results of the peptides binding to the intimal surface of the WHHL and NZW rabbit aortas is that the binding is widely variable except in the case of peptide 9. Peptide 9 consistently shows staining on the WHHL aortic tissue. Furthermore, peptide 9 binds regions of plaque in a dose-dependent manner. Peptide 9 also shows some slight staining on the NZW tissue; however, this staining is shown to represent a consistent background staining, because the overall staining does not increase with an increase in
the peptide concentration. Other peptides that show above-average reproducible staining on WHHL tissue are peptides 3 and 4. The figures that follow will show the results for these three peptides of interest.

Figures 3.5 and 3.6 below show the results of the binding assay for peptide 9 (C-terminal FLAG sequence) from two experiments performed on aortic tissue samples from two distinct WHHL rabbits. The sections shown in (a) – (d) were incubated with a 1X concentration of peptide 9 (~ 10 µg/mL) and assayed according to Section 2.6.2, while those shown in (e) – (f) are the control sections that were not incubated with peptide and were used to determine whether anti-FLAG-HRP bound to the tissue itself. Note that the controls in Figure 3.5(e) and 3.6(f) show some regions of brown (positive) staining, but the other control sections are clear. Relative to the staining seen in the control sections, the sections showing staining in (a) – (d) in both figures show more total staining, and the staining is very distinct. It is important to note, however, that there are some tissue sections that were incubated with peptide 9 that did not show staining. The fact that the tissue is heterogeneous could account for this observation. Overall, peptide 9 was the peptide that showed the most consistent binding to the WHHL tissue throughout the experiments.
Figure 3.5: **Result of WHHL assay with peptide 9 (exp201).** (a) – (d): Sections incubated with peptide 9 (1X concentration). (e) – (f): Anti-FLAG-HRP control sections. Note that these results are from a different experiment than those shown in Figure 3.6.
Figure 3.6: Result of WHHL assay with peptide 9 (exp223). (a) – (d): Sections incubated with peptide 9 (1X concentration). (e) – (f): Anti-FLAG-HRP control sections. Note that these results are from a different experiment than those shown in Figure 3.5.
Figures 3.7 and 3.8 below show the results of the binding assay for peptides 3 and 4 (both N-terminal biotinylation) performed on sections from the same WHHL rabbits as in Figures 3.5 and 3.6, respectively. The sections shown in (a) and (b) were incubated with peptide 3, and those shown in (c) and (d) were incubated with peptide 4. The sections were assayed according to Section 2.6.2. The sections shown in (e) – (f) are the control sections that were not incubated with peptide and were used to determine whether SA-HRP bound to the tissue itself. Note that the controls for the biotinylated peptides, (e) – (f), show much less staining than the control for peptide 9, which is detected by anti-FLAG-HRP. Peptide 4 shows good staining in Figure 3.7(d), whereas peptide 3 yields the best staining in Figure 3.8(b). However, the corresponding tissue samples in Figure 3.7(c) for peptide 4 and Figure 3.8(a) for peptide 3 show very little, if any, brown staining. This demonstrates the variability of peptide binding not only between different rabbits, but also the variability within each animal. This variability between different animals was present throughout the experiments for all of the peptides that showed binding activity, except for peptide 9.
Figure 3.7: Result of WHHL assay with peptides 3 and 4 (exp201). (a) – (b): Sections incubated with peptide 3. (c) – (d): Sections incubated with peptide 4. (e) – (f): SA-HRP control sections.
Figure 3.8: Result of WHHL assay with peptides 3 and 4 (exp223). (a) – (b): Sections incubated with peptide 3. (c) – (d): Sections incubated with peptide 4. (e) – (f): SA-HRP control sections.
When peptide 9 was first assayed on NZW control tissue at the 1X concentration, it was seen to bind with a light, distinct staining pattern. Peptides 3 and 4 showed no binding to NZW tissue. These results are shown in Figure 3.9 below. Figure 3.9 shows the results of two separate experiments on two NZW rabbits. The images in (a), (c), and (e) show sections incubated with peptide 3, peptide 4, and the SA-HRP control, respectively and are from one experiment (exp199). The images in (b) and (d) show sections from a different experiment (exp214) incubated with peptide 9 (1X concentration), and the image in (f) shows the anti-FLAG control. Note that the sections incubated with peptide 9 in (b) and (d) show a subtle, yet distinct, brown staining pattern on the surface, while the control in (f) shows no staining. Therefore, peptide 9 initially showed targeting to the aortic surface on both the WHHL and NZW animal models. Peptides 3 and 4 show no staining on the NZW aorta samples as seen in images (a) and (c).
Figure 3.9: **Result of NZW assay with peptides 3, 4, and 9 (exp199, exp214).** (a), (c), (e): Sections incubated with peptide 3, peptide 4, and SA-HRP control, respectively, from exp199. (b), (d), (f): Sections incubated with peptide 9 at 1X concentration (b) and (d), and anti-FLAG control, from exp214.
The fact that peptide 9 showed slight staining on the NZW tissue was originally interpreted to mean that peptide 9 binds the surface of rabbit aorta in a nonspecific manner. However, recent results show that when the concentration of peptide 9 is increased 10-fold (≈ 100 µg/mL), the staining shown on the WHHL tissue increases dramatically, while the staining on the NZW tissue remains the same as the staining seen with the 1X concentration. These results are shown below in Figure 3.10. The top row shows the results on WHHL tissue while the bottom row is NZW tissue. The images in (a) and (d) show the staining caused by the 1X concentration of peptide 9 on WHHL and NZW tissue, respectively (from Figures 3.5 and 3.9). The images in (b) and (e) show the staining of peptide 9 when the concentration is increased to the 10X concentration on WHHL and NZW tissue, respectively. Note the drastic difference in staining seen on the WHHL tissue when the concentration of peptide 9 is increased 10-fold from (a) to (b). However, there is little difference in the staining on the NZW tissue when the concentration of peptide 9 is increased by the same magnitude from (d) to (e). The control sections in (c) and (f) are from the 10X experiment and show no staining. These results indicate that peptide 9 targets plaque-rich WHHL tissue and that the staining on the NZW tissue is a consistent background staining.
Figure 3.10: **Comparison of 1X and 10X concentrations of peptide 9 on WHHL and NZW tissues.** Top row: WHHL tissue. Bottom row: NZW tissue. (a) – (c): 1X concentration, 10X concentration, and anti-FLAG-HRP control on WHHL tissue. (d) – (f): 1X concentration, 10X concentration, and anti-FLAG-HRP control on NZW tissue.
After the initial assays indicated that peptides 9, 3, and 4 were potential targeting candidates to WHHL rabbit aorta, peptides were synthesized that had randomly scrambled amino acid sequences corresponding to the original peptides. Peptide 9 (TQTPIKHHLLKE-FLAG) was randomly reordered to ILTHEHTLPKQ-FLAG, which will be referred to as peptide 9R (peptide 43 in Table 3.1). Similarly, peptides 3R and 4R were synthesized as Biotin-PPEEWSPVTHLL (peptide 44 in Table 3.1) and Biotin-RNPGTYHIALG (peptide 45 in Table 3.1), respectively. The peptides were randomized such that no two amino acids in the original sequence appeared sequentially in the new (random) sequence. If the peptides are targeting plaque based on the specific order of the primary amino acid sequence, then the randomly sequenced peptides should show decreased binding activity. The following figures show the results of the binding assays comparing peptides 9, 3, and 4 to their scrambled counterparts, 9R, 3R, and 4R from one experiment on a WHHL rabbit (exp249).
Figure 3.11: Result of WHHL assay with peptide 9R (randomized peptide 9 sequence) from exp249. (a): Section incubated with peptide 9. (b): Section incubated with peptide 9R. (c): anti-FLAG-HRP control sections.
Figure 3.12: Result of WHHL assay with peptides 3R and 4R (randomized peptide 3 and 4 sequences) from exp249. (a) – (b): Sections incubated with peptide 3 and 3R, respectively. (c) – (d): Sections incubated with peptides 4 and 4R, respectively. (e): SA-HRP control sections.
Figure 3.11 shows the results for peptides 9 and 9R, along with the corresponding control sections. The staining of peptide 9 in (a) is stronger than the staining seen from peptide 9R in (b), indicating that the primary amino acid sequence of peptide 9 is responsible for recognizing the surface of the aorta. Note that the control for this experiment (c) shows very slight staining, but it is much less than the staining present in (a). Figure 3.12 shows the results for peptides 3 and 3R, 4 and 4R, and the corresponding control sections. Both randomized sequences in (b) and (d) show much less staining compared to the original sequences shown in (a) and (c), which is evidence that peptides 3 and 4 also bind due to the configuration of amino acids in the original sequences. Also, most of the control sections in (e) for peptides 3 and 4 show no staining.

To demonstrate that the location of positive peptide binding is in fact the intimal layer of the aorta, several pieces of positively stained WHHL aorta with peptide 9 from exp249 were sectioned for histological analysis. A Hoechst stain was performed on the tissue sections in order to visualize nuclei near the intima. The following figures show the results of this experiment. Figure 3.13 shows a section of a plaque-laden region of WHHL tissue stained with peptide 9. The stain is clearly localized to the intimal layer, with no infiltration into the plaque region. Furthermore, the Hoechst stain in image (b) suggests an intact endothelial layer, but this should be confirmed with an endothelium-specific marker. Both images in Figure 3.13 are 400X magnification.
Figure 3.13: **Binding of peptide 9 to WHHL aorta is localized to the intimal layer of the aorta.** (a) The positive brown staining is clearly present only on the intimal surface with no infiltration into the plaque region. (b) Hoechst stain suggests presence of an intact endothelium along with positive brown staining of peptide 9 on the surface. Both images are 400X magnification.

It was also observed that most of the staining of peptide 9 is present on intima covering regions of plaque but not over non-plaque regions. This result is shown in Figure 3.14. The image in (a) is taken over a plaque-rich region, while that in (b) is taken from a region with no observable plaque. A distinct difference in surface morphology distinguishes plaque from non-plaque regions. The intima covering non-plaque regions shows the characteristic wavy appearance seen in (b). Note that brown staining is only seen over the plaque-rich region in (a).
Figure 3.14: **Binding of peptide 9 to WHHL aorta is seen over plaque-rich regions.** *(a)* The positive brown staining is seen over intima covering plaque. *(b)* *No stain* is present over the intima covering a region with no plaque.

It is important to note that staining was present on regions where the endothelium was *removed*. The endothelium could be removed by the detection assay or by the sectioning, and it is possible that the endothelium is *degraded in vivo*. Also, although most staining was present on intima covering plaque, some staining was seen on intima covering a small region that had no observable plaque, and *staining* was absent in a small area covering a plaque-rich region of the section. This indicates that the presence of plaque is neither a necessary nor sufficient condition for the *binding* of peptide 9 to the intimal aortic *surface*, which follows logically from the observation that peptide 9 shows slight background staining on NZW samples that have no observable plaque.

To *demonstrate* the variability in binding of the remaining peptides, the following figures (Figures 3.15 – 3.18) show staining of WHHL tissue samples from all of the
peptides that showed any positive activity from four different experiments. Between five and seven tissue samples were incubated with all peptides in each experiment.

Figure 3.15: Binding of peptides to WHHL aorta sections (exp201). (a) – (d): Peptides 1, 10, 20, and anti-FLAG-HRP control, respectively. (e) – (i): Peptides 6, 7, 11, 24, and SA-HRP control, respectively.
Figure 3.16: Binding of peptides to WHHL aorta sections (exp217). (a) – (d): Peptides 2, 14, 16, and anti-FLAG-HRP control, respectively. (e) – (i): Peptides 6, 7, 12, 13, and SA-HRP control, respectively.
Figure 3.17: Binding of peptides to WHHL aorta sections (exp219). (a) – (c): Peptides 24, 28, and SA-HRP control, respectively.

Figure 3.18: Binding of peptides to WHHL aorta sections (exp223). (a) – (e): Peptides 34, 37, 38, 39, and SA-HRP control, respectively.
Figure 3.15 shows the results from exp201. Peptides 1, 6, 7, 10, 11, 20, and 24 showed staining on one tissue sample. The controls for this experiment in (d) and (i) show no staining. Figure 3.16 shows tissue samples stained with peptides 2, 6, 7, 12, 13, 14, and 16 from exp217. The controls shown in (d) and (i) show minor staining. Note that this is the second observation of slight staining by peptides 6 and 7 on one of the several tissue samples that were incubated with peptide. Figures 3.17 and 3.18 demonstrate that peptides 24, 28, 34, 37, 38, and 39 all showed some staining on tissue samples from exp219 and exp223. The controls for these experiments in Figure 3.17(c) and Figure 3.18(e) show no staining. It is important to note that when these peptides demonstrated staining, the staining was only seen on a fraction of the tissue samples that were incubated with the same peptide; typically only one or two samples showed staining. Therefore, these peptides may be potential targeting agents to plaque, but peptide 9 yielded the most consistent binding of all peptides assayed, and peptides 3 and 4 showed above-average staining consistency. A summary of these results is shown in Table 3.2.

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<td>Sequence</td>
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<td>CKSDANSHC-FLAG</td>
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<tr>
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<td>Biotin-CAPGPPDSK</td>
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<td>14</td>
<td>CKQSPPSMC-FLAG</td>
</tr>
<tr>
<td>24</td>
<td>Biotin-SSSKMGAHQLP</td>
<td>16</td>
<td>KSLSRHDHHH-FLAG</td>
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</table>

Table 3.2: **Peptides that showed positive binding.** Results are summarized for all experiments, showing all peptides that displayed positive binding, excluding 9, 3, and 4.
3.2.3 Frozen Tissue Assay Results

Several of the synthesized peptides were assayed for binding to frozen WHHL and NZW tissue as described in Section 2.6.3. Of the peptides that have been tested, peptide 9 shows the most significant and consistent binding to the tissue samples. Figure 3.19 shows the results of the binding assay for peptides 9 and 9R, and for the anti-FLAG-HRP control on WHHL tissue. The image shown in (a) is the result of incubating the tissue section with peptide 9. Note that the assay yields strong brown staining on the surface. The scrambled version of peptide 9, peptide 9R, was incubated with the tissue section shown in (b). Clearly, there is no staining seen from peptide 9R, further supporting the hypothesis that the specific amino acid sequence of peptide 9 is responsible for binding. The anti-FLAG control is shown in (c). This also shows no brown staining. All images are 400X magnification.

Figure 3.19: Binding of peptide 9 to frozen WHHL aorta tissue sections. (a) Peptide 9 stains the intimal surface. (b) Peptide 9R shows no binding. (c) Anti-FLAG control. All images are 400X magnification.
3.2.4 ApoE Knockout Mice Results

As of the writing of this thesis, very few initial experiments have been performed on the ApoE knockout mouse system, and no results will be shown as figures. Peptides 7 and 9 have been tested for binding using in vivo circulation of peptide, followed by the removal of the aorta and assaying for activity as previously described (Section 2.6.4). These experiments did not yield positive results. Peptides 7 and 9 have also been conjugated to iron oxide nanoparticles, injected into mice, and imaged in vivo using MRI to try to detect a change in signal between pre- and post-contrast particle injection. The results of these experiments are currently inconclusive. These experiments will be continued in the future using the other synthesized peptides.

3.3 Pull-Down Experiment Results

Initial experiments were performed prior to the pull-down experiment to identify some characteristics of the system used to isolate the protein to which peptide 9 binds. The initial experiments were an attempt to locate the presence of the iron oxide nanoparticles, alone and after conjugation with peptide, on the gel after electrophoresis. This is important because if the particles appear in the same region as the isolated protein of interest (the “pulled-down” protein), then there is a fundamental limitation of the system. Two gels were run with different loading conditions, and samples were prepared both with heating at 70°C for 10 minutes (recommended by Invitrogen) or without heating. One loading condition was the NuPAGE system described in Section 2.7. This system includes reducing agent in the sample preparation as well as antioxidant in the running buffer. The second loading system was a homemade buffer of 10% glycerol,
0.1% bromophenol blue without reducing agents in the sample preparation and without antioxidant in the running buffer. The results of this experiment are shown in Figure 3.20.

Figure 3.20: **Experiment to determine location of nanoparticles on the NuPAGE gel.** Four lanes (4 – 7) are shown flanked by standard ladders (3 and 8). Lanes 4 and 5 are the SA-coated nanoparticles conjugated to peptide 9 from heated and non-heated samples, respectively. Lanes 6 and 7 are SA-coated nanoparticles without conjugated peptide from heated and non-heated samples, respectively. (a) Result for loading buffer containing 10% glycerol without reducing agent. (b) Result for NuPAGE loading buffer containing reducing agent. Note that for the conjugated particles (lanes 4 and 5), no streak is observed on the gel using either system.

The image in (a) is the result using the homemade loading system (10% glycerol), while the image in (b) shows that of the NuPAGE loading system. Lanes 3 and 8 in both systems are the protein standard ladder, which ranges from 3 kDa (bottom) to 200 kDa (top). Lanes 6 and 7 in both systems are the SA-coated iron oxide nanoparticles (heated
and not heated, respectively), and lanes 4 and 5 are the SA-coated particles conjugated with peptide 9 (heated and not heated, respectively). The gel in (a) has a slight tear near the top, but this did not affect the interpretation of the results. Notice that in both systems, the particles (6 and 7) show up as a smear in the 50 – 100 kDa region of the gel. The most important conclusion from this experiment is that the conjugated particles (4 and 5) do not show up as a smear on the gel in either system, regardless of heating treatment. Based on this observation, the pull-down experiments were performed using the NuPAGE system with reducing agent in the sample preparation, antioxidant in the running buffer, and heating the samples at 70°C for 10 minutes, as recommended by the supplier.

The first isolation experiment (drw5) was performed as described in Section 2.7. After the first magnetic separation, the supernatant was poured off and stored in buffer containing protease inhibitors at 4°C. This supernatant was run on the gel along with the isolate from the magnetic separation that was collected after the washing steps. The supernatant should theoretically contain several proteins that were not bound to the SA/peptide 9 conjugated particles and would provide a reference for the isolated protein band. The result of the first isolation attempt is shown in Figure 3.21. Lanes 3 and 6 are the protein standard ladder, lane 4 is the isolate from the magnetic separation, and lane 5 is the supernatant from the first magnetic separation step. The molecular sizes in the regions of interest are shown on the figure.
Figure 3.21: **Result of the initial pull-down experiment (drw5)**. Lanes 3 and 6 are the protein standard ladders. Lane 4 is the sample containing the *isolate from the magnetic separation*. *Lane 5 is the sample containing* the supernatant from the first magnetic separation step, which should contain several proteins.

The results indicate a protein band near the 6 kDa region in the pull-down sample (lane 4), but this protein was also present in the *lane containing the supernatant* (lane 5). The major concern from the result of lane 5 is that there does not appear to be a wide variety of proteins, which indicates that the membrane protein extraction steps were not very successful. The supernatant should contain all the extracted proteins from the *WHHL aorta* that were not magnetically separated, but only one band is present in lane 5. As of the writing of this thesis, the mass spectroscopy results for the 6 kDa band were *not* available. However, the band present below the 3 kDa standard in lane 4 is peptide 9, as
demonstrated in a separate experiment (data not shown). *This indicates that excess peptide was present in the pull-down sample.*

After the initial pull-down experiment *(drw5)*, an experiment was performed to attempt to isolate more membrane protein from the aorta tissue sample *(drw6)*. The impetus for this experiment was that the supernatant *(lane 5, Figure 3.21)* showed only one protein band, but should have contained several bands of membrane proteins. To increase total protein content isolated from the resected tissue, a hand-held tissue homogenizer was used to blend the tissue. The Mem-PER extraction kit was then used to isolate membrane proteins from the homogenized sample. *Protease inhibitors were added to the reagents at each step in the protein isolation in order to enhance the extraction.* A sample of the raw tissue homogenate was collected after blending the tissue sample, and a sample of the final step in the membrane protein isolation was collected to run on a gel. The results of these samples after electrophoresis are shown on the gel in *Figure 3.22*. Lanes 1 and 9 are the protein standard ladders. Lane 4 is the sample from the raw tissue homogenate without added reagents from the Mem-PER extraction kit. *Lane 5 is the sample containing the isolated membrane proteins.* Approximate molecular weights are indicated to the left of the figure. The other lanes are samples that are unrelated to *this discussion*. 
Figure 3.22: Result of the attempt to improve membrane protein extraction efficiency (drw6). Lanes 1 and 9 are the protein standard ladders. Lane 4 is the sample containing the raw tissue homogenate without added reagents. Lane 5 is the sample containing the isolated membrane proteins from the Mem-PER extraction kit. The other lanes contain samples unrelated to this experiment. Approximate molecular weights are indicated on the figure.

The results in Figure 3.22 show that the raw tissue homogenate (lane 4) yielded several proteins in the 40 – 200 kDa range. However, the sample from the isolated membrane proteins obtained from the extraction kit shows only two small bands near 45 and 60 kDa. This indicates that a large loss of protein occurs using the Mem-PER extraction kit, and a larger tissue sample may be required to obtain enough membrane proteins for the pull-down experiment.
CHAPTER 4
DISCUSSION

4.1 Peptide Binding Assays

The results presented in Section 3 were intended to present an overview of the positive binding outcomes from the selected peptides. Unfortunately, the binding assays performed on the lipid-loaded HAECs (Section 3.2.1) yielded no positive binding, despite the demonstration that the cells accumulated the LDL, which mimics the early state of human atherosclerosis [44]. However, the HAEC binding assays were only performed with peptides 1 – 33 (excluding 8), because the remaining peptides had not yet been synthesized at the time of the experiments. After peptides 1 – 33 (excluding 8) showed no positive results, it was decided that continuing the assays with the remaining peptides would not be an efficient use of resources. Culturing the cells takes much time and effort, and the binding assays are also extremely time-consuming. Due to the fact that the rabbit tissue binding assays were showing much more promising results, full attention was placed on those assays. This is not meant to suggest that the remaining peptides would probably not demonstrate binding to the HAECs; rather, they should be tested in future studies.
Of all the peptides tested on the resected aortic tissue samples from WHHL and NZW rabbits, the most consistent targeting agent to plaque is peptide 9, while peptides 3 and 4 are other above-average consistency targeting candidates. This does not imply that the other peptides listed in Table 3.1 did not show any binding capability. Actually, several peptides showed some distinct binding in some of the experiments (Figures 3.15 – 3.18, Table 3.2), but the binding results for these peptides were variable. There are several possible explanations for the observed variability in the binding of these peptides. The most obvious explanation is the heterogeneity of the tissue. The gross morphology of the WHHL aortas showed some very large regions of plaque with smaller plaque-free regions interspersed. It is possible that while randomly selecting tissue samples from the aortas, some peptides were exposed to tissue samples composed primarily of plaque-free regions. Another possibility that can account for some variability is that the plaque itself is heterogeneous, and some regions of the plaque may not express enough of the compounds that are targeted by certain peptides. Thus, if a peptide targets a specific region of the plaque that is not present in the randomly selected tissue samples, the binding assay will appear negative. Finally, it is possible that the peptides may degrade over time, since they are stored in solution. Therefore, the peptides listed in Table 3.1 are all potential plaque targeting agents that exhibit variable binding results when assayed according to the described experiments.

However, peptide 9 showed the most consistent staining ability across all WHHL animals and within the majority of the tissue samples from each experiment (Figures 3.5 and 3.6). Furthermore, when the concentration of peptide 9 was increased 10-fold, the
result of the binding assay to the WHHL tissue showed a dramatic increase in staining (Figure 3.10). The slight staining of peptide 9 seen on the non-diseased NZW rabbit tissue (Figure 3.9) is apparently due only to background binding as seen in the result shown in Figure 3.10; that is, a 10-fold increase in the concentration of peptide 9 does not result in increased binding to NZW tissue. This indicates that peptide 9 may target a marker on aortic intima that is specific for plaque.

It has also been demonstrated that peptide 9 binds to WHHL tissue based on its primary amino acid sequence. The scrambled variant of peptide 9 (peptide 9R) showed much less binding than peptide 9 (Figure 3.11), indicating that the correct peptide sequence is vital for binding. The same phenomenon was observed for peptides 3 and 4. When randomly scrambled versions of these sequences were assayed for binding, they showed less staining compared to the original sequences (Figure 3.12). These results should be verified using 10X concentrations of the scrambled peptides. If the 10X concentrations of the scrambled versions show the same amount of staining as the 1X concentrations, then the conclusion that the binding is due to the primary amino acid sequence will be strengthened greatly.

Peptides 3 and 4 also show staining on WHHL tissue (Figures 3.7 and 3.8) that appeared more consistent than the other peptides tested with the binding assay system. Furthermore, these peptides do not exhibit staining on the NZW tissue (Figure 3.9). This indicates that peptides 3 and 4 may be potential targeting agents specific to plaque. However, it is important to note that peptides 3 and 4 showed somewhat variable staining capabilities on the WHHL tissue. Throughout the different experiments, these peptides

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sometimes showed very good staining, but in others seemed to not bind at all. Therefore, while peptides 3 and 4 may be targeting agents specific to some marker of plaque, it is possible that the binding affinities of these peptides are less than the affinity of peptide 9. Perhaps the amino acid sequences of peptides 3 and 4 could be optimized to enhance binding to plaque.

In addition to the proposed mechanisms of variability discussed above, another important consideration regarding the peptides is the modification made to each peptide in order to assay for binding. Specifically, the biotinylation at the N-terminal could interfere with binding if the recognition site of the peptide is near the N-terminal. Similarly, the addition of the FLAG sequence to the C-terminal could interfere with binding if the recognition site is near that terminal. The modifications were chosen randomly for each peptide, but the location of the modification (N- or C-terminal) was dictated by the ease of synthesis, recommended by GenScript. It may be beneficial to add a linker sequence, such as a glycine-glycine linker, between the modification and the peptide sequence to allow for improved recognition of the peptide to its target. Furthermore, a peptide expressed via phage display will be fused to the phage protein coat at its C-terminal, leaving the N-terminal end of the peptide relatively free with respect to its spatial orientation. Thus, modifications made to the free peptides at the N-terminal may have resulted in negative binding results, because the binding site has a high probability of becoming blocked if the N-terminal is modified without a linker.

In addition to demonstrating the consistency of the binding capability of peptide 9, it was also shown that peptide 9 specifically targets the intima of the WHHL aorta.
(Figures 3.13 and 3.14). Staining was seen on fixed tissue sections in the intima over regions of plaque. Most of the intima over non-plaque areas were shown to have no staining. However, the presence of plaque was neither a necessary nor sufficient condition for staining, as a small region of staining was also present over a non-plaque region, and staining was absent in an area where plaque was seen. This is not surprising when considering the result that peptide 9 binds NZW tissue as shown by the presence of some background staining. The dose-independent staining on the NZW tissue demonstrates that peptide 9 can bind some regions of tissue where there is no observable plaque. However, the fact that peptide 9 binds WHHL tissue in a dose-dependent manner indicates that peptide 9 may have a preference for binding a marker that is specific for plaque. Furthermore, when peptide 9 binds plaque, it binds to the intimal surface of the aorta. This is a very positive result in the sense that if peptide 9 is used as the targeting agent for an image-enhancing nanodevice, the contrast agent will be delivered to the regions of plaque on the surface of the aorta and will most likely not be internalized. By minimizing internalization, the potential of toxicity is reduced.

The results of the frozen tissue section assays support the fact that peptide 9 binds WHHL aortic tissue samples (Section 3.2.3). However, these assays are primarily a screening technique to identify which of the synthesized peptides are most likely to bind the resected tissue sections, because binding to frozen tissue may be drastically different than binding to fresh tissue. Also, no conclusions can yet be made for the ApoE knockout mice experiments (Section 3.2.4), primarily because the experiments have not been repeated. Peptide 9 was originally discovered by biopanning in a rabbit system, and
therefore it may not bind the mouse model. However, screening the remaining peptides in this system could yield positive results.

4.2 Pull-Down Experiment

Currently, the protein to which peptide 9 binds has not yet been isolated. However, several important experiments have been performed to better understand the system developed to isolate this protein. It has been shown that the NuPAGE system for gel electrophoresis under reducing conditions is ideal for eliminating a smear on the gel that could be caused by the magnetic particles. The Mem-PER protein extraction kit has been used to successfully isolate membrane proteins from fresh tissue samples; however, the efficiency of the extraction may be lower than what is required to detect the pulled-down protein. According to Pierce, it is possible to scale-up the protocol by using more tissue and a proportional amount of reagents. In the work of Liu et al. [42], a similar pattern to that observed on the gel in Figure 3.22 was shown from the protein extraction of the endothelial cells from mice. Therefore, the extraction procedure is most likely effective, but the protein of interest may not present in a large enough quantity to detect.

Another issue that deserves careful consideration is the fact that the C-terminal FLAG modified version of peptide 9 was shown to bind the tissue samples, not the N-terminal biotinylated version that is used in the pull-down experiment. If the N-terminal modification of the peptide reduces the ability of peptide 9 to bind its target protein, then the pull-down experiment will not be successful unless much more of the protein can be extracted from the tissue. Thus, a logical next step would be to try a C-terminal biotinylated peptide 9 with a linker between the peptide and the biotin as the targeting
agent. This would allow the free N-terminal end to bind the target protein, and the linker between the peptide and the biotin would allow the SA-coated nanoparticles to have better access to the biotin.

4.3 Conclusions and Recommendations for Future Work

The results presented in this work meet most of the goals specified in Section 1.6. Several novel targeting agents have been identified by biopanning on the WHHL rabbit model of atherosclerosis with phage displayed peptide libraries. The peptides selected for synthesis were assayed on lipid-loaded HAECs, WHHL and NZW tissue samples, and ApoE knockout mice in vivo. Peptide 9 was shown to be the most consistent targeting agent to plaque, and peptides 3 and 4 show above-average consistency of binding on the WHHL tissue compared to the other peptides. Furthermore, peptide 9 was shown to specifically bind plaque in a dose-dependent manner. However, the molecular cognate of peptide 9 has not been successfully isolated from the aortic tissue. This should be the most immediate focus of future work and should continue with the recommendations made in Section 4.2.

Another immediate goal should be to continue screening of peptide affinity to the ApoE knockout mouse model. It is possible that some of the peptides in Table 3.1 are able to bind plaque in the mouse model but not in the WHHL model (or potentially both models). Also, these animals are smaller than the rabbits and smaller quantities of reagents (peptides, iron oxide nanoparticles) are required for detection assays, and recently MR images of the ApoE mice aortas have been successfully acquired.
Long term goals of this project should be as follows:

- Demonstrate in vivo binding capability of peptides by conjugating them to USPIOs and imaging using MR.
- Demonstrate disease state specificity of the targeting agents (early vs. late disease).
- Determine the prognostic significance of targeted USPIOs for plaque vulnerability.
- Develop a targeted nanotherapeutic based on novel targeting agents and current clinical therapeutics.

Completing these goals will result in a suite of biological nanodevices that have the capability to target vulnerable plaque. These devices could be used as a screening tool for populations at high risk for acute coronary syndromes and vulnerable plaque rupture. Therapies exist to treat plaque [45], [46], but these are useless if the patient suffers a coronary event resulting in death. Targeting image-contrast agents to vulnerable plaque will identify which patients need the most urgent and aggressive therapies. Ideally, the targeting agent will be specific for a particular grade of plaque and may even include a therapeutic to be delivered to the disease site, thus potentially reducing the need for surgical intervention. While this is a noble goal with many technical problems yet to be mastered, it is only a matter of time before cardiovascular nanodevices will be able to detect and treat vulnerable plaque.
APPENDIX A

SEQUENCES ISOLATED FROM BIOPANNING
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<th>Peptide Sequence</th>
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Table A.1: Sequences obtained from lipid-loaded HAEC panning experiments. More than 60 peptides were isolated de novo from panning on lipid-loaded HAECs with phage displayed peptide libraries. The 7-mers shown are in reality flanked by cysteine residues on either side of the sequence, but the abbreviation, C, has been omitted for clarity.
### Table A.2: Sequences obtained from WHHL rabbit biopanning experiments

More than 200 peptides were isolated de novo from biopanning on WHHL rabbits with phage displayed peptide libraries. The 7-mers are in reality flanked by cysteine residues on either side of the sequence, but the abbreviation, C, has been omitted for clarity.

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LIST OF REFERENCES


