Cholesterol binding activity of ApoAI mimetic peptide L4F

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The University of Toledo
A Thesis

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Cholesterol Binding Activity of ApoA-I Mimetic Peptide L4F

By

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Submitted as partial fulfillment of the requirements for

The Master of Science in Medicinal and Biological Chemistry

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An abstract of

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According to the current WHO statistic, cardiovascular diseases are the leading cause of deaths all over the world. Over 1.7 million people have died due to cardiovascular disorders like myocardial infarction, coronary artery diseases and stroke. A common condition underlying almost all of these disorders is an excessive accumulation of lipids- more specifically cholesterol. Thus, managing the levels of cholesterol can prove to be a very effective strategy in preventing or treating several cardiovascular disorders. ApoA-I mimetic peptides are promising candidates in this respect. L4F is a synthetic apoA-I mimetic peptide containing 18 L- amino acid residues of which four are phenylalanine and hence the name. It has been found to be capable of reducing lesions due plaque formation in atherosclerosis in mice. Animal models and cell studies have demonstrated the capability of L4F to reduce the expression of inflammatory cytokines
IL-1, IL-6 and TNFα. L4F is also found to be capable of binding oxidized lipids. Although a lot is known of the biological activity of L4F, very little is known about its structure and how that relates to its varied functions. It is unknown how or if L4F is capable of binding cholesterol in solution state. Thus, our research is a step forward in that direction.

We have successfully determined that L4F can bind cholesterol in solution state. The binding was detected using an enzyme assay. The principle of the assay is such that it can detect cholesterol that is in solution and available for the enzyme to detect. Thus L4F is capable of solubilizing cholesterol and making it available in solution. L4F showed detergent-like action where it solubilized cholesterol from a pellet of cholesterol and made it detectable by the enzyme. We were also successful in determining the time course of binding. The binding or complex formation starts almost instantaneously but L4F-cholesterol bound complex starts to fall apart over a period of time. The maximum binding was detected at 3hrs after which it gradually decreases and by 12hrs when barely any could be detected. This property of L4F is favorable for developing it as a drug molecule.

We made efforts to express the peptide in bacterial cells to obtain isotopically labeled peptide. This isotopically labeled peptide would eventually be used for structural analysis by Hydrogen-Deuterium Exchange using NMR or Mass spectrometry. The gene construct (oligo) for the peptide was designed and primers were designed for PCR amplification of the double stranded oligos for insertion into vector for expression. PCR products were obtained successfully.
The expression of isotopically labeled peptide and structural analysis of L4F are the ultimate goals of this research and other lab members will continue efforts in that direction.
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Chapter 1

Introduction

Cardiovascular diseases are a group of disorders of the heart and blood vessels. They include atherosclerosis, coronary artery disease (CAD), myocardial infarction, stroke etc. According to the WHO, cardiovascular diseases (CVDs) are the number one cause of death globally: more people die annually from CVDs than from any other cause[1]. An estimated 17.3 million people died from CVDs in 2008, representing 30% of all global deaths[1, 2] The WHO estimates that almost 25 million people will die from cardiovascular diseases by the year 2030. Thus, there is no doubt that efforts need to be made in order to prevent, treat and cure cardiovascular diseases.

There are several factors that are identified as potential causes of various heart diseases. Some of the most common factors are high blood pressure, smoking, obesity, type 2 diabetes, insulin resistance and high amounts of fatty acids and cholesterol in the blood. Of all the cardiovascular diseases, atherosclerosis is possibly the biggest threat since it can indirectly lead to other disorders like ischemia, coronary artery disease, peripheral arterial disease as well as chronic kidney disease. It can lead to serious and life threatening problems including heart attack and stroke[3]. One of the leading causes of atherosclerosis is high amount of lipids and cholesterol in the blood. The level of cholesterol circulating in the blood has been shown to have a direct correlation to the risk of development of atherosclerosis and subsequently other cardiovascular diseases [4].
In the wake of this health crisis and a global demand for an effective remedy for cardiovascular disorders, efforts are being made to develop a drug molecule that effectively prevents and/or treats this class of disorders. L4F is one such molecule developed to better target the root cause of atherosclerosis and related diseases. It is an apoA-I mimetic synthetic peptide that has been shown to be capable of binding cholesterol and other oxidized lipids. It also plays a role in preventing inflammation and hence prevents formation of atherosclerotic plaque. Thus, through its activity, it has the potential to be used in the treatment of lipid-related cardiovascular disorders. Through its cholesterol scavenging action, L4F can also be used for prophylaxis in susceptible individuals. In order to understand how L4F can effectively treat cholesterol-related disorders in the body, let us first try to understand the function and regulation of cholesterol in the body.

**Cholesterol biosynthesis**

Cholesterol is an extremely important biological molecule. Chemically, it is a polycyclic compound with the molecular formula C$_{27}$H$_{46}$O. It belongs to the class of sterol lipids or steroids. It plays many important roles in membrane structure. It is also a precursor for the synthesis of steroid hormones, vitamin D in skin and bile acids. It is synthesized *de novo* in the body as well as obtained from dietary sources. The typical human body contains about 900mg of cholesterol- 200-300 of which comes from diet and the rest is synthesized in the body[5]. Slightly less than half the cholesterol in the body is derived from biosynthesis in the liver, intestine, cytoplasm and endoplasmic reticulum.
Figure 1 Biosynthesis of cholesterol

As depicted in the flowchart above, cholesterol biosynthesis is a multistep process. There are close to 30 different enzymes required for these reactions. It starts with acetyl coenzyme A reacting with acetoacetyl coenzyme A in presence of an enzyme called HMG CoA synthase to yield HMG CoA, which is then reduced to Mevalonate with the help of enzyme HMG CoA reductase. Mevalonate then undergoes several more steps to finally yield cholesterol. The most effective of all the drugs currently being used for the treatment of hypercholesterolemia are the statins[6]. Statins lower the biosynthesis of cholesterol by inhibiting the enzyme HMG CoA reductase [7].
1.2 Cholesterol Transport

The cholesterol exists as free cholesterol when it is first synthesized in the hepatic cells in the liver. In circulating blood, cholesterol is carried as cholesteryl ester which is an inactive form. These are formed by esterification of cholesterol molecule with fatty acid molecule[8]. Cholesteryl esters are a component of entities called lipoproteins.

Lipoproteins are multi-molecular complexes that transport and deliver cholesterol and other components to the target tissue. The principal plasma lipoproteins are chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL)[8]. Functions of each one of these lipoproteins can be generalized in the table below.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Function</th>
<th>Transports cholesterol from</th>
<th>Transports cholesterol to</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>Mobilize dietary lipids</td>
<td>Enterocytes via lymph</td>
<td>Peripheral tissues</td>
</tr>
<tr>
<td>VLDL</td>
<td>Transport endogenously derived triglycerides and cholesterol</td>
<td>Hepatic tissue</td>
<td>Adipose/ muscle tissue</td>
</tr>
<tr>
<td>LDL</td>
<td>Primary carrier of cholesterol derived from liver</td>
<td>Hepatic tissue</td>
<td>All tissues</td>
</tr>
<tr>
<td>HDL</td>
<td>Pickup cholesterol, accumulate cholesteryl esters and once filled, return to liver</td>
<td>Peripheral tissues, blood</td>
<td>Hepatic tissue</td>
</tr>
</tbody>
</table>

Table 1 Types and functions of plasma lipoproteins
Essentially, the cholesterol from diet is absorbed in the gut where it is emulsified with bile salts and released into the blood as nascent chylomicrons. These chylomicrons transport exogenous/dietary cholesterol to liver, adipose, cardiac and skeletal muscles. The chylomicron remnants after fat digestion are cleared in the liver. The cholesterol synthesized in the liver is carried by VLDL, which is converted to LDL in the peripheral tissue. This LDL binds to the LDL receptors to deliver cholesterol in the liver as well as peripheral cells [9]. HDL is synthesized in the liver and acts as scavenger collecting free cholesterol from cells and converting it to esters before returning it to the liver for disposal. The major catabolic route for excretion of cholesterol is by its conversion into excretable bile acids[10].

1.1 Cholesterol biosynthesis regulation

Synthesis and utilization of cholesterol must be tightly regulated in order to prevent accumulation or abnormal deposition within the body. Of particular importance clinically is the abnormal deposition of cholesterol and cholesterol-rich lipoproteins in the coronary arteries. There are three distinct mechanisms by which the cellular supply of free cholesterol is maintained in the body[11].

1. Regulation of HMG CoA Reductase (HMGR) activity and levels.

2. Regulation of excess intracellular free cholesterol through the activity of acyl-CoA: cholesterol acyltransferase (ACAT).

3. Regulation of excess intracellular free cholesterol level via LDL receptor-mediated uptake and HDL-mediated reverse transport.
HMG CoA reductase is responsible for the synthesis of mevalonate, which is an important precursor of cholesterol. The activity of this enzyme is regulated in the body through a multivalent feedback mechanism. Two of these important factors are- a.) LDL binding to the LDL receptors in peripheral cells to deliver cholesterol and b.) negative feedback through the mevalonate synthesized[12]. Statins, which are HMG CoA reductase inhibitors, act by decreasing the hepatic secretion and increasing the catabolic rates of VLDL and LDL [13]. The enzyme Acyl CoA cholesterol acyl transferase or ACAT is responsible for esterification of free cholesterol that is absorbed from the small intestine. Regulation of excess free cholesterol can be mediated by regulating the activity of ACAT. Thus ACAT has direct effect in blocking absorption of cholesterol in small intestine as well as in reducing secretion of VLDL in hepatocytes[14]. Another way of regulating cholesterol biosynthesis is by LDL receptor-mediated uptake of excess of free cholesterol or by HDL mediated reverse transport.

1.3 HDL and reverse cholesterol transport (RCT)

High Density Lipoprotein (HDL), commonly known as “good cholesterol” has been demonstrated to be anti-atherogenic[15]. They are the smallest of lipoprotein particles and also the densest since they contain the highest protein to cholesterol content. HDL circulates in the bloodstream and is responsible for extracting cholesterol from body tissues and transporting it to the liver for recycling or excretion. Nascent HDL particles are discoidal consisting of a phosphatidylcholine bilayer and a protein shell which shields the hydrophobic lipid tails. The cholesterol and phospholipids from the cell membranes dissociate and are picked up by particles called pre β- HDL[16]. Pre β HDL attaches to the cell surface via special receptors and transfers the cholesterol to HDL. The enzyme
lecithin cholesterol acyltransferase (LCAT) now binds to the maturing HDL particle. As cholesterol accumulates within HDL, the conformation of LCAT changes with it and is activated. The LCAT converts amphipathic cholesterol to hydrophobic cholesterol esters to avoid back transport. The esters are deposited into the lipid tails of the lipid bilayer of HDL. This changes the shape of HDL from discoidal to spherical at which point the cholesterol collection ceases and the mature HDL particle is recognized by the liver[16, 17]. This process is known as reverse cholesterol transport (RCT). A schematic view of the process of reverse transport is depicted in the following figure.

![Figure 2 Reverse Cholesterol Transport](image)

1.4 Apolipoprotein A-I (apoA-I)

Apolipoprotein A-I or apoA-I is the primary protein constituent of HDL. It is also found in small amounts in chylomicrons and VLDL. The two major sites of synthesis of apoA-I are liver and intestine. The apoA-I derived from the intestine is carried by chylomicrons
to the plasma where they are transferred immediately to HDL. That synthesized in the liver is in association with VLDL [18]. Being the primary protein constituent in the dense protein rich HDL, apoA-I plays following important roles[19]-

- Defining the size and shape of HDL particles
- Solubilizing the lipid contents of HDL
- Removing cholesterol from peripheral cells
- Activating the enzyme LCAT
- Delivering the resulting cholesterol esters to liver for disposal or recycling

ApoA-I has also been found to play an important role in modulating the factors involved in inflammation. It is now known to inhibit cytokine production upon contact with stimulated T cells. The low levels of HDL associated apoA-I in cases of chronic inflammation indicate that apoA-I might be the missing link between infection and chronic inflammation [20].

Structurally, apoA-I is a single polypeptide made of 243 amino acids. The sequence is now known[18]. apoA-I has an important structural feature. A large portion of the protein structure is a series of tandemly repeated 22 amino acid segments which contain proline residues almost exclusively. These segments have shown to have helical structure with amphipathic character[21]. This amphipathic helix is known to play important role in several functions of apoA-I like lipid binding and activation of LCAT[22].

1.5 ApoA-I mimetic peptides and L4F
The crucial role that apoA-I plays in regulation and metabolism of cholesterol lead to the development of a series of synthetic peptide mimetics of apolipoprotein apoA-I. L4F is
one such synthetic peptide developed in 1980s. It is a synthetic moiety based on the sequence of a short segment of apoA-I which is amphipathic in nature and is thought to have a helical structure. It was synthesized using L amino acids and modified to contain four phenylalanine residues and hence the name L4F. The primary structure of L4F is as follows-

Asp-Trp-Phe-Lys-Ala-Phe-Tyr-Asp-Lys-Val-Ala-Glu-Lys-Phe-Lys-Glu-Ala-Phe

The peptide has both its terminals modified. The N terminal is carboxylated and the C terminal is amidated. Thus the peptide is represented as follows


L4F is capable of binding both oxidized as well as unoxidized lipids. It is in fact known to have greater affinity for lipids than apoA-I itself [23]. L4F being an apoA-I mimetic, can enhance the ability of HDL-cholesterol to protect LDL-cholesterol from oxidation. This leads to a reduced production of LDL induced monocyte chemoattractant protein 1 (MCP1) in endothelial cells which in turn reduces atherosclerosis [24]. Thus it can convert HDL-cholesterol from pro inflammatory to anti-inflammatory. This explains the anti-atherogenic properties of L4F. Inflammatory disorders are also thought to play a role in the pathogenesis of myocardial and endothelial dysfunction in diabetes [25]. L4F is found to cause a decrease in the levels of inflammatory cytokines IL-1, IL-6 and TNF-α along with improved vascular reactivity [26, 27]. Taking all these effects into consideration, it is realized that L4F is a potential therapeutic agent for several lipid related or lipid dependent disorders. Thus, it is important to investigate further into the mechanism of action and functions of L4F. Studies identifying the possible targets of L4F
along with the mechanism that L4F uses to bring about these effects are essential. The
next chapter provides a brief review of the research carried out so far along with the aims
and objectives of our research.
Chapter 2

Literature Review

2.1 Development of apoA-I mimetics

The efficacy of apoA-I in animal models and preliminary human studies made it an important therapeutic candidate[28]. The initial promise during clinical trials faded eventually as the cost of synthesis and difficulty in administration increased. ApoA-I is a 243 amino acid long peptide which made it expensive to synthesize. Also, being a peptide, it had to be given intravenously to avoid digestion/degradation by peptic acids and this made its administration cumbersome. As a result, the labs of Segrest Jere and Anatharamaiah G.M designed an 18 amino acid peptide called 18A. The peptide was called 18A because it formed a class A amphipathic helix. A class A amphipathic helix is defined as an α-helix which has opposing polar and non-polar faces that are oriented along its long axis. The peptide 18A did not have any sequence homology with apoA-I but it mimicked the amphipathic helices in apoA-I[29]. The stability of peptide 18A was improved when an acetyl group was added at the N terminal and an amide group at the C terminal. Adding these end groups proved useful in increasing the helicity of the peptide and caused an increased self-association among peptide molecules [30]. The peptide 18A was now called Ac-18A-NH$_2$ or 2F since it contained 2 phenylalanine residues at the hydrophobic face [31]. Many 2F variants were later developed and these were named based on the number and position of phenylalanine residues. They were tested for biological activity in cocultures of human artery wall cell cultures and mouse models of
atherosclerosis [32]. Of all the compounds tested, both 4F and 5F were found to be active in artery wall cell cultures as well as prevent lesions in mouse models. The peptide 3F with a phenylalanine residue on the polar face was found to be ineffective. However, the same peptide with a phenylalanine on the non-polar face, called 3F-2 was found to be very effective in cell based assays. All these minor but important changes cannot be explained effectively by the assumed amphipathic α helical structure. Hence, studies to characterize the structure of the peptide are essential.

2.2 Development and study of L4F

The peptide 4F was synthesized using both L and D amino acids. When synthesized with L amino acids- L4F was rapidly degraded in the GI tract of mice upon oral administration. However, when synthesized with D amino acids- D4F, it was intact in plasma and showed significant reduction in atherosclerotic lesions in both LDL receptor-null mice on Western diet as well as apoE-null mice on chow diet [33]. The plasma cholesterol or the HDL level did not show any alteration in either D4F or L4F administration. However, the anti-inflammatory properties of HDL were studied using a cell-based assay and showed a significant improvement. Plasma tested from hemodialysis patients after incubation with L4F showed marked increase in the anti-inflammatory activity of HDL and sharp decrease in the pro-inflammatory activity of LDL[34]

Inflammation is now known to play at all stages of atherosclerosis. It was capable of causing an increase in the pre-β HDL, enriched in paraoxonase activity. L4F also enhanced reverse cholesterol transport from macrophages. Thus, the key to its activity could be the lipid shuttling effect of the peptide rather than binding to a specific receptor. It has also shown the capability of improving insulin sensitivity and adiponectin levels in
obese mice [26]. In a recent study by Vecoli et al L4F has shown to prevent myocardial and coronary dysfunction in diabetic mice [35]. In a study carried out by Van Lenten et al, the binding of L4F to several oxidized and unoxidized lipids was analyzed by immobilizing the peptide on a chip and detecting binding using Surface Plasmon Resonance (SPR) [23]. The $K_D$ of binding was found to be in nanomolar range for most oxidized lipids.

2.3 Objectives of our research

Even though a lot is now known about the activity of L4F through both animal and cell studies, the underlying mechanism of these activities is yet to be determined. For understanding these, a structural study is needed. This requires reconstitution of L4F with cholesterol in solution. There is also a lack of understanding regarding the solution state behavior of the peptide L4F. Studies have either determined only its biological effects or its binding after it has been immobilized on a chip in order to bind. There seems to be a gap in our knowledge of the behavior of this peptide with lipids in solution state. Thus, what we are interested in determining is the interaction between the peptide L4F and lipid-cholesterol specifically- in solution state. The ultimate goal of our research is to carry out structural studies for the analysis of mechanism of action of L4F. We intend to achieve this using the method of Hydrogen-Deuterium Exchange studies either by NMR or Mass spectrometry. Studies of L4F and cholesterol binding in solution are a necessary step towards achieving these long-term goals. The primary objectives of the study described in this paper are as follows

1. **Objective I- To express the peptide in bacterial cells in order to obtain isotopically labeled L4F for structural studies using NMR.**
2. **Objective II- Study the binding of L4F to cholesterol in solution state.**

The following chapters describe our efforts towards fulfilling these objectives along with a brief discussion of results and conclusion.
Chapter 3

Materials and Methods

3.1 Materials

3.1.1 L4F peptide

The peptide was obtained as a gift sample from Dr. Nader Abraham’s lab, Department of Physiology and Pharmacology. L4F is an 18 amino acid containing peptide. The N terminal of the peptide has an acetyl modification and the C terminal has an amide modification. Following is the sequence of the peptide-


The molecular weight of the peptide was calculated to be 2310. It was confirmed using mass spectrometry. The peptide was freely soluble in water as well as HEPES. It was obtained in powdered form and was stored in the freezer at -80°C at all times. The peptide was protected from moisture by adding desiccant to the container. In order to avoid subjecting the peptide to freeze-thaw cycles, a 100uM stock solution of peptide in HEPES buffer and distilled water was made. It was then divided into several 100uL aliquots and stored at -20°C.

3.1.2 Cholesterol

Cholesterol [CAS no.57-88-5] was purchased from Sigma Aldrich [Product no. C8667]. Molecular weight of cholesterol is 386.7 and melting point is 147-150°C [36].
Cholesterol is practically insoluble in water (less than 0.2 mg/100 ml of water), slightly soluble in alcohol (1.29% w/w at 20 °C) more soluble in hot alcohol (2.6mg/ml at 50°C)[37]. Thus stock solutions of varying concentrations of cholesterol in ethyl alcohol were made. They were divided in aliquots of 10ml and stored at -20°C. The cholesterol stock solutions were protected from light [36].

3.1.3 Amplex® Red Cholesterol Assay Kit

In order to determine the interaction between the peptide and cholesterol, the enzyme assay kit was used. The kit was purchased from Invitrogen (Life Technologies) [catalog number A12216]. It contains Amplex® Red reagent, DMSO, horseradish peroxidase, hydrogen peroxide, cholesterol esterase, cholesterol oxidase, cholesterol standard reference, resorufin fluorescence standard and 5x reaction buffer containing Triton®X-100 and cholic acid. The kit provides a sensitive, rapid and simple fluorimetric method of detecting very low concentrations of cholesterol along with cholesteryl esters. Allows multiple time point measurements.[38].

3.1.4 GEV1 vector

GEV1 is an expression system designed specifically for easy expression of isotopically labeled peptides for NMR analysis[39]. The vector was purchased from Addgene [no.12615]. It was obtained as a stab culture. After overnight incubation, bacterial growth was visible both in the puncture and on the surface of the agar. These bacteria were then plated on fresh LB agar plate with ampicillin. The bacterial cells were harvested after overnight incubation in liquid LB and plasmid was isolated using miniprep kit.
3.1.5 DNA oligos and primers for PCR

Based on the amino acid sequence of the L4F peptide, a DNA template oligo was designed for in vitro expression of the peptide. In order to create multiple copies, PCR technique was used. For that, primers were also designed for PCR. Both the oligo and primer were purchased from Eurofins MWG Operon.

3.1.6 QIAprep Spin Miniprep Kit

In order to make copies of vector and the complete reconstructed plasmid used for the expression of the isotopically labeled peptide, the plasmids were isolated using miniprep kit from Qiagen [catalog no. 27104].

3.1.7 QIAquick Gel Extraction Kit

In order to ligate the vector (GEV1) with the insert (Oligos), isolation of vector fragments and insert fragments from gel after gel electrophoresis was carried out. For this purpose, the gel extraction kit was purchased from Qiagen [catalog no. 28704].

3.1.8 NheI and XhoI restriction enzymes.

Both vector (GEV1) and insert (Oligos) have two restriction sites built in their sequences. Thus, the vector and insert can both be cut to cleave or produce sticky ends at these sites using the restriction enzymes. NheI and XhoI were the two restriction used for this purpose. NheI (catalog no. R0131L) and XhoI (catalog no. R0146L) were purchased from New England BioLabs.
3.1.9 Bovine Serum Albumin (BSA)

BSA was used to prevent adhesion of the restriction enzymes to tubes and pipette surfaces. It also stabilizes the enzymes during incubation. BSA was purchased from New England BioLabs [catalog no. B9001S].

3.1.10 T4 DNA Ligase

The enzyme used for the ligation of vector (GEV1) and insert (Oligo), the enzyme T4 DNA ligase was used. T4 DNA ligase was purchased from New England BioLabs [catalog no. M0202T].

3.1.11 Taq DNA polymerase

For making copies of the oligo/ gene construct that codes for the peptide, PCR technique was used. For this purpose, the enzyme Taq DNA polymerase was used which helps in adding the nucleotide bases to the elongating strand during PCR. Taq DNA polymerase was purchased from New England BioLabs [catalog no. M0273S]

3.1.12 Competent cells

One shot®TOP10 cells were purchased from Invitrogen (Life Technologies). They were stored at -80°C at all times. Competent cells are required for transformation and cloning of plasmids.

3.1.13 Agarose (High resolution/ Molecular biology grade)

Agarose was purchased from Sigma-Aldrich [CAS no. 9012-36-6]. It was used for identification and separation of various fragments of DNA by gel electrophoresis.
3.2 Methods

3.2.1 Assay of Cholesterol in Solution

An easy and sensitive method for analyzing the interaction between peptide L4F and cholesterol is to assay the increase of cholesterol concentration in solution due to cholesterol solubilizing activity of L4F. To assay this cholesterol in solution, an enzyme based assay that can determine the concentration of cholesterol in solution was used. The Amplex® Red Cholesterol assay kit used for our study provides a simple fluorometric method for sensitive quantitation of cholesterol using a fluorescence microplate reader.

3.2.1.1 Preparation of solutions before assay[40]

3.2.1.1.1 Amplex® Red reagent stock solution

A 20mM stock solution of Amplex® Red reagent was prepared by allowing one vial of Amplex® Red reagent (Component A) and the DMSO (Component B) to warm to room temperature. Immediately prior to use, the contents of the vial of Amplex® Red reagent (1 mg) in were dissolved in 200µL DMSO. The stock solution was stored frozen at ≤–20°C, protected from light.

3.2.1.1.2 Reaction Buffer working solution

A 1x working solution of Reaction Buffer was prepared by adding 2.5 mL of 5 X Reaction Buffer stock solutions (Component E) to 10 mL of deionized water (dH2O).
3.2.1.1.3 Horseradish peroxidase (HRP) stock solution

A 200 U/mL stock solution of horseradish peroxidase (HRP) was prepared by dissolving the contents of the vial of HRP (Component C) in 1 mL of 1X Reaction Buffer. After use, the remaining solution was divided into small aliquots and stored frozen at ≤–20°C.

3.2.1.1.4 H₂O₂ working solution

A 20mM H₂O₂ working solution was prepared by diluting the ~3% H₂O₂ stock solution (Component D) into the appropriate volume of dH₂O. The 20mM H₂O₂ working solution is not stable and was used promptly.

3.2.1.1.5 Cholesterol oxidase stock solution

A 200 U/mL solution of cholesterol oxidase was prepared by dissolving the entire vial of cholesterol oxidase (Component F) in 250µL of 1X Reaction Buffer. After use, the remaining solution was divided into small aliquots and stored frozen at ≤–20°C.

3.2.1.1.6 Cholesterol esterase stock solution

A 200 U/mL stock solution of cholesterol esterase was prepared by dissolving the entire vial of cholesterol esterase (Component G) in 250µL of 1X Reaction Buffer. After use, the remaining solution was divided into small aliquots and stored frozen at ≤–20°C. Cholesterol esterase is added to the reaction to allow detection of cholesterol in the form of cholesteryl esters.

3.2.1.1.7 Resorufin stock solution

A 2mM stock solution of resorufin was prepared by adding 1 mL dH₂O directly to the vial of resorufin solid (Component I). This solution was used to prepare a standard curve.
to determine the moles of product produced in the Amplex® Red reaction. Resorufin stock solution was stored frozen at \( \leq -20^\circ C \), protected from light.

### 3.2.1.2 Preparing cholesterol standard[40]

A cholesterol standard curve needs to be plotted for each time the assay is performed. The assay is sensitive in detecting up to 30µM cholesterol. Thus the appropriate amount of 5.17mM cholesterol reference standard (Component H) was diluted into 1X Reaction buffer to produce cholesterol concentrations of 0 to 30µM. 1X Reaction Buffer without cholesterol was used as a negative control. A volume of 50µL was used for each reaction.

### 3.2.1.3 Preparing Amplex Red reagent working solution[40]

The working solution of 300µM Amplex® Red reagent contains 2 U/mL HRP, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase. It was prepared by adding 75µL of Amplex® Red reagent stock solution, 50µL of the HRP stock solution, 50µL of the cholesterol oxidase stock solution, and 5µL of the cholesterol esterase stock solution to 4.82 mL of 1X Reaction Buffer. This 5 mL volume is sufficient for \( \sim 100 \) assays.

The working solution was divided into 1ml aliquots and stored frozen and protected from light at -20°C.

### 3.2.1.4 Experimental Protocol [40]

Following is the general protocol or sequence of steps for the assay. Several modifications were made to the assay at various stages of experimentation. These modification or changes are described in detail wherever relevant in the results and discussion section.
1. A positive control was prepared by diluting the 20mM H$_2$O$_2$ working solution to 10µM in 1X Reaction buffer.

2. 50µL of the cholesterol-containing samples was diluted in 50µL of 1X Reaction Buffer. A volume of 50µL of this diluted sample was used for each reaction.

3. 50µL of the diluted samples and controls were pipetted into separate 1.5ml microcentrifuge tubes.

4. The reactions were started by adding 50µL of the Amplex® Red reagent working solution to each microcentrifuge tube containing 50µL of the samples and controls.

5. The reactions were incubated for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence could be measured at multiple time points to follow the kinetics of the reactions.

6. The fluorescence was measured in a fluorescence microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm.

7. Correction for background fluorescence was made for each sample reading by subtracting the values derived from the no-cholesterol control from.

3.2.2 Expression of isotopically labeled peptide

Many studies have been carried out on using Nuclear Magnetic Resonance as a technique for structural studies. We are interested in determining the structure and binding interaction of the peptide L4F with cholesterol. In order to facilitate the efficient study of this interaction by NMR, it is advantageous to express the peptide in a host (usually bacteria) for easy and inexpensive labeling with $^{15}$N and reconstituting a complex of cholesterol and L4F.
The peptide is a small molecule with a proposed alpha helical structure. It is found to be freely soluble in water and HEPES buffer. It is fairly stable in solution, which is a prerequisite for NMR studies. For carrying out the NMR studies, we need to first express the peptide in suitable bacterial host cells so that we can label them with $^{15}$N.

### 3.2.2.1 Polymerase Chain Reaction

After a careful study of all the parameters for primer design, a suitable primer and template were designed for the purpose of PCR. PCR was carried out to amplify and make copies of the gene construct for the L4F peptide, which would then be used for expressing the peptide in the bacterial cells.

**Protocol:**

1. Due to the difficulties in pipetting small volumes of enzyme, Taq DNA Polymerase was diluted in Diluent F or 1X PCR reaction buffer. 1 $\mu$L of Taq DNA Polymerase was added to 4$\mu$L of diluent and 1 $\mu$L of that mixture is used for the final reaction. Enzyme diluted in Diluent F was stored at -20°C for future use.
The following 50μL reaction was prepared in a 0.5ml PCR tube on ice.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Standard Taq reaction buffer (10x)</td>
<td>5</td>
<td>1x</td>
</tr>
<tr>
<td>2. Deoxynucleotide solution</td>
<td>1</td>
<td>200μM</td>
</tr>
<tr>
<td>3. Upstream primer- Primer1 (10μM stock)</td>
<td>0.5-2.5</td>
<td>0.1-0.5μM</td>
</tr>
<tr>
<td>4. Downstream primer- Primer 2 (10μM stock)</td>
<td>0.5-2.5</td>
<td>0.1-0.5μM</td>
</tr>
<tr>
<td>5. DNA template</td>
<td>0.5-1.5</td>
<td>0.1-1ng/ml</td>
</tr>
<tr>
<td>6. Taq Polymerase</td>
<td>0.2</td>
<td>0.02unit/μL</td>
</tr>
<tr>
<td>7. DEPC treated water</td>
<td></td>
<td>Bring reaction to a final volume of 50μL</td>
</tr>
</tbody>
</table>

Table 2 PCR reaction setup.

1. The reaction was gently mixed and spun down in microcentrifuge.

2. Cycling Conditions for a Routine PCR Reaction were as follows
3.2.2.2 Preparing LB agar plates with ampicillin

1. 100ml of sterilized LB broth was taken in a clean glass flask.

2. 1.5gm of Agar was weighed accurately and added to the flask containing LB broth.

3. The solution was heated in the microwave until the entire agar melted. Short bursts of heat were given by heating for 10 seconds and then swirling the flask for mixing and heating for another 10 seconds.

4. After the flask began to cool down, at about 40°C, 500uL of ampicillin stock (100mg/ml) was added to the flask.

5. The flask was swirled to allow adequate mixing of the antibiotic. Bubble formation was avoided.

6. 20 polystyrene petri dishes were labeled indicating that Ampicillin was added and the date.

<table>
<thead>
<tr>
<th>Process</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>85°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>30 cycles</td>
<td>85°C</td>
<td>15-30 seconds</td>
</tr>
<tr>
<td></td>
<td>45-65°C</td>
<td>15-60 seconds</td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>1 minute per kb</td>
</tr>
<tr>
<td>Final Extension</td>
<td>68°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Table 3 Cycling conditions for PCR
7. Approximately 20ml of LB agar was poured in each plate.

8. The lids on the plates were placed and they were allowed to cool for about 30 minutes or until solidified.

9. The plates were stored in plastic bags sealed with parafilm at 4°C.

**3.2.2.3 Streaking and isolating bacteria on an LB Agar plate**

1. A LB agar plate with antibiotic (Ampicillin) was obtained.

2. The working area in the laminar airflow cabinet/hood was sterilized using isopropyl alcohol.

3. The bacteria growing within the punctured area of the stab culture or the top of the glycerol stock were touched using a sterile loop.

4. The bacteria were gently spread over a section 1 of the plate, as shown in the diagram below, to create streak 1.

5. Using a fresh new sterile loop, the bacteria were dragged through streak 1 and spread to section 2 to create streak 2.

6. Using a fresh new sterile loop, the bacteria were dragged through streak 2 and spread to section 3 to create streak 3.

7. The plate was incubated overnight at 37°C.

8. Single colonies were visible on the plate in the morning.

9. If the growth on the plate was too dense, new plates were re-streaked using the same method described above to get single colonies.
10. A liquid culture of the bacteria was prepared before harvesting them for plasmid prep by taking a sterile 10ml tube.

11. 5mL of liquid media- LB broth was added to the tube.

12. 50uL of ampicillin stock was added to it and stirred.

13. Using a sterile pipet tip, a single bacterial colony was picked from the plate and transferred immediately to the liquid media.

14. The tube was sealed loosely with a cap to allow flow of oxygen.

15. It was placed in shaker incubator at 37°C for 5-7 hours.
3.2.2.4 Plasmid miniprep [41]

1. The bacterial liquid culture was pelleted in a small microcentrifuge tube at 14000 rpm for 10 minutes.

2. The pellet cells were resuspended in 250uL of Buffer P1. Mixed by inverting the tube a couple of times.

3. 250uL of Buffer P2 was added and mixed thoroughly by inverting the tube up and down for 5-6 times until the solution becomes clear. This lysis reaction was not allowed to exceed 5 minutes.

4. 350uL of Buffer N3 was added and mixed immediately and thoroughly. A uniform cloudy precipitate was visible.

5. Tube was centrifuged for 10 minutes at 13000 rpm in a microcentrifuge.

6. The supernatant from step 5 was applied to the QIAprep spin column by decanting or pipetting. Centrifuged for 30-60seconds and the flow-through was discarded.

7. Adding 0.75ml Buffer PE and centrifuging for 30-60 seconds washed the QIAprep spin column. The flow-through was discarded and the tube was centrifuged for another minute to remove residual buffer.

8. The QIAprep column was placed in a clean 1.5ml microcentrifuge tube. To elute DNA, 50uL Buffer EB or water was added to the center of the QIAprep spin column and was allowed to stand for 1 minute and then centrifuged for 1 minute.

9. The tube was labeled and placed at -20°C until further use.
3.2.2.5 Restriction digestion of plasmid/ PCR product

The restriction maps of both vector and insert indicate that the enzymes that can be used to specifically cut GEV1 (vector) and Oligo for L4F (insert) are NheI and XhoI.

1. A diagnostic digest was carried out first to ensure that the plasmid obtained from plasmid miniprep contains the fragment of interest. It was carried out on a small scale and analyzed using gel electrophoresis.

2. After this confirmation, a scale up digest was carried out to isolate the fragment of interest after restriction digestion.

3. In a 1.5ml microcentrifuge tube, all the components were mixed as per the table below.

4. The digestion reaction was run for 4-12hrs in a water bath at 37°C.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Plasmid DNA(from miniprep or PCR)</td>
<td>5.0</td>
</tr>
<tr>
<td>2. Restriction enzyme NheI</td>
<td>0.5</td>
</tr>
<tr>
<td>3. Restriction enzyme XhoI</td>
<td>0.5</td>
</tr>
<tr>
<td>4. NEB Buffer 2</td>
<td>2.0</td>
</tr>
<tr>
<td>5. BSA (10X)</td>
<td>2.0</td>
</tr>
<tr>
<td>6. Sterile water</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 4 Diagnostic digest
### 3.2.2.6 Gel electrophoresis

1. **Material used:**
   - Agarose
   - Distilled water
   - 1X TAE buffer
   - Ethidium bromide stain
   - Loading dye (Bromophenol blue)

2. **Buffers and solutions prepared before electrophoresis:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Plasmid DNA (from miniprep or PCR)</td>
<td>100.0</td>
</tr>
<tr>
<td>2. Restriction enzyme NheI</td>
<td>5.0</td>
</tr>
<tr>
<td>3. Restriction enzyme XhoI</td>
<td>5.0</td>
</tr>
<tr>
<td>4. NEB Buffer 2</td>
<td>20.0</td>
</tr>
<tr>
<td>5. BSA (10X)</td>
<td>20.0</td>
</tr>
<tr>
<td>6. Sterile water (only if DNA is inadequate)</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Table 5 Scale-up digests
• 10X TAE buffers- The 10X stock solution of buffer was made by dissolving the following in 1L of distilled water: 48.8g of Tris Base, 11.4ml of Glacial Acetic Acid, 3.7gm of EDTA disodium salt.
• 1X TAE buffer- 100ml of 10X buffer stock was diluted to 1L with distilled water.
• 1.2% Agarose gel- 1.2gm of Agarose was dissolved in 10ml of 1X TAE buffer.

3. Sample preparation for gel electrophoresis

• In a fresh new microcentrifuge tube, 5µL of the restriction digestion product was added to 5µL of loading dye (Bromophenol blue). In case of scale up digest, to the entire digestion product in the tube, an equal amount of loading dye was added before loading onto the gel for electrophoresis.

4. Protocol

• The agarose gel was heated in the microwave until it became liquid. Adequate amount of the liquid agarose was poured in the gel-casting tray containing the comb for wells. The liquid agarose was allowed to cover up to 2/3rd of the comb teeth.
• The liquid was allowed to sit for 15-20minutes until it set into a gel.
• Once the gel had solidified, the comb was gently removed so that the wells are not disturbed or punctured.
• The gel tray was placed in the electrophoresis box filled with 1X TAE buffer. It was made sure that the tray was placed in such a way that the wells in the gel were on the side of the negative electrodes of the electrophoresis box.
• In a fresh new microcentrifuge tube, 5µL of marker or DNA ladder was added to 5µL of loading dye and mixed gently by pipetting up and down.
• 10µL of marker was added in the first well from the left in the gel.
• In the remaining wells, the sample containing loading dye was added making sure the pipet tip did not puncture the well in the agarose gel while loading the samples.
• The electrodes were connected to the power supply and the voltage was set between 100 to 150V.
• The gel was allowed to run for 40 to 60 minutes until the Bromophenol blue band was about 4-6 cms away from the top of the gel. It was made sure that the band did not run off from the bottom of the gel.
• The power supply was turned off and the gel was removed from the electrophoresis box.
• The gel was then removed from the gel cast and placed in a separate staining box, which contained 5µL of ethidium bromide in water. Gloves were worn at all the stages.
• The gel was stained for 15 to 20 minutes and then observed under the UV-trans illuminator for locating the different bands.

3.2.2.7 DNA extraction protocol[42]
1. In order to isolate the DNA of interest from the gel after electrophoresis, the gel was placed on the UV trans illuminator and the lamp was turned on. Protective gloves were worn and UV shied was used to limit exposure to UV as much as possible.
2. With a sharp blade, precise cuts were made around the band of interest on the gel making sure to not include the marker/ ladder DNA in that piece of gel.
3. The UV was turned off as soon the band was cut.
4. An empty tube was weighed and its weight was recorded. The gel band was
   transferred into the tube. The tube was weighed again and the difference between two
   weights was calculated in order to determine the weight of the gel slice.
5. 3 volumes of buffer QG were added for each volume of gel for example- 300µL of
   buffer were added for each 100mg of gel.
6. The tube was incubated at 50°C for 10 minutes or until the gel slice had melted. The
   tube was swirled intermittently.
7. 1 volume of isopropanol was added for each volume of gel making sure that the color
   of the solution containing gel and QG buffer did not change.
8. The contents were added to a QIAquick spin column in the provided 2ml collection
   tube.
9. The tubes were centrifuged for 30-60 seconds in order to allow the DNA to bind to
   the column containing silica.
10. The flow-through was discarded and the column was placed in a clean fresh
    microcentrifuge tube.
11. 0.75ml of buffer PE (washing buffer) was added to the tube and centrifuged for 30-
    60seconds. The flow-through was discarded.
12. The tube was centrifuged again for 30-60 seconds to get rid of any residual washing
    buffer.
13. The QIAquick spin column was placed in a new microcentrifuge tube and 50µL of
    elution buffer EB was added to elute the DNA from the column. The column was
    spun for 30-60 seconds and the spin column was then discarded.
14. The flow-through now contained the eluted DNA from the column. This was stored at -20°C until further use.

3.2.2.8 Ligation of vector and insert

After the isolation and purification of both vector (GEV1) and insert (Oligos), it was made sure the amount of insert was at least twice as much as the amount of vector. This was to drive forward the ligation reaction between the vector and insert and avoid the vector or insert themselves. The ligation reaction was set up with two other control reactions. All ligation reactions were carried out overnight by incubating the samples at 37°C.

In three separate microcentrifuge tubes, the reactions were set up as per the tables below:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vector (GEV1)</td>
<td>8 (at least 50ng)</td>
</tr>
<tr>
<td>2. Insert (Oligo)</td>
<td>9 (at least 100ng)</td>
</tr>
<tr>
<td>3. T4 DNA ligase</td>
<td>2</td>
</tr>
<tr>
<td>4. 2X ligation buffer</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 6 Ligation reaction
Control 1 (No insert)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vector (GEV1)</td>
<td>8 (at least 50ng)</td>
</tr>
<tr>
<td>2. Distilled water</td>
<td>9</td>
</tr>
<tr>
<td>3. T4 DNA ligase</td>
<td>2</td>
</tr>
<tr>
<td>4. 2X ligation buffer</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 7 Ligation control 1

Control 2 (No ligase enzyme)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vector (GEV1)</td>
<td>8 (at least 50ng)</td>
</tr>
<tr>
<td>2. Insert (Oligo)</td>
<td>9 (at least 100ng)</td>
</tr>
<tr>
<td>3. Distilled water</td>
<td>2</td>
</tr>
<tr>
<td>4. 2X ligation buffer</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 8 Ligation control 2

3.2.2.9 Transformation of competent cells

In order to check if the ligation reaction had worked, we needed to transform competent cells with the ligated plasmid. For this, all three of the ligation reactions mentioned above were used for transformation of competent cells.

Protocol: [43]

1. The vials containing the ligation reactions were centrifuged briefly and placed on ice.
2. One 50µL vial of One Shot® cells was thawed in ice for each ligation/transformation.
3. To transform, both of the following were used:
• 1 to 5µL of a ligation reaction

• 10pg supercoiled plasmid (i.e. pUC19 as the transformation control)

4. 5µL of DNA i.e. each one of the ligation reactions were added directly to each of the three vials of the competent cells and mixed by tapping gently. The remaining ligation reactions were stored at -20°C.

5. The cells were incubated on ice for 30 minutes.

6. Then the tubes were incubated for exactly 30 seconds in the 42°C water bath without mixing or shaking.

7. The vials were removed from the 42°C bath and quickly placed on ice.

8. 250µl of pre-warmed SOC medium was added to each vial. Good sterile technique was practiced to avoid contamination.

9. The vials were placed in a microcentrifuge rack on its side and secured with tape to avoid loss from the vials. The vials were shaken at 37°C for exactly 1 hour at 225 rpm in a rotary shaker-incubator.

10. 10µL to 50µL from each transformation vial was spread on separate, labeled LB agar plates. Two different volumes were plated to ensure that at least one plate has well-spaced colonies. For the transformation control, 50µL of pUC19 control were plated.

11. The plates were inverted and incubated at 37°C overnight.

12. Single colonies were selected for further analysis by plasmid isolation.
Chapter 4

Results and Discussion

4.1 Mass spectrometry

Before starting any experimentation with the L4F peptide, which was obtained as a gift sample, we wanted to confirm the peptide’s identity, its molecular weight as well as make sure that it was fairly pure. For this purpose, we carried out a Mass spectrometry. The experiments were carried out on an electrospray mass spectrometer with quadrupole mass analyzer. ESI-MS (Electrospray ionization Mass Spectrometry) is capable of generating highly charged ion species of peptides. It is typically produced by multiple protonation (positive ion mode) or deprotonation (negative ion mode) of the species. In the native state, peptides and proteins are folded. As a result, some amino acid residues could be buried away from the solvent and hence unavailable for protonation or deprotonation. In order to improve protonation or deprotonation, the intermolecular bonds need to be broken or the peptides and proteins need to be unfolded. This can be achieved using a 1:1 mixture of water and an organic solvent. We carried out MS in positive ion mode. Hence, the ions produced would be due to protonation of basic amino acid residues. In case of the peptide L4F, there are four lysine residues which can be potentially protonated. The formic acid added assures protonation of the lysine side chains without unfolding of the peptide. Thus we expected ions with +2, +3 +4 charge.

The MS experiment was carried out under the guidance of Dr. Dragan Isailovic in his lab, in the Department of Chemistry. The sample for MS consisted of 100µL acetonitrile+ 100µL of 1mg/ml L4F in distilled water+ 0.2µL of formic acid. Electrospray ionization
MS was carried out in the positive ion mode. The instrument used was LCQ DECA XP plus with ESI and ion trap. The flow rate for sample injection was 5µL/min. Nitrogen was used as sheath gas for nebulization. The ESI voltage was 4.7kV. The scans were set to measure for the mass range of 150-2000. The mass spectrum obtained can be seen below

5. **Figure 4 Positive ion ESI for L4F peptide**

The calculated weight of L4F was 2310.61[47] The mass range for the instrument did not allow us to measure ions above the m/z of 2000. As a result, the molecular ion peak cannot be seen in this spectrum. However, the doubly charged ion peak was visible at m/z 1156.27 with a relative abundance of 67%. The most abundant of all the ions were triply charged ions at m/z 771.20 with a relative abundance of 100%. The peak at 578.73
represents the ions with a +4 charge. However the relative abundance for these ions is only 50%.

The mass spectrum confirmed that the sample of peptide was fairly pure and that the molecular weight of L4F was 2310.61, as calculated. It also confirmed the identity of peptide indicating that it has not undergone any modifications or degradation.

4.2 Fluorescence Binding Assay

Several studies have been carried out in order to determine the interaction of the apoA1 mimetic peptide L4F with oxidized phospholipids. Not many studies have tried to determine the interaction of L4F with cholesterol. One study investigating the binding of cholesterol to L4F was carried out by Van Lenten et al. The technique used in this study to analyze this binding was surface plasmon resonance which immobilizes the peptide on a chip and measures the binding as a solution of cholesterol flows over it. Thus it was not a study carried out in solution state in the true sense. In our research, we were interested in finding out the how the peptide binds or interacts with cholesterol in a solution phase. For this purpose, we decided to use a fluorescence based assay which enables the quantification of the bound and hence solubilized cholesterol in a solution containing L4F and cholesterol.

Cholesterol itself does not exhibit any fluorescence. The peptide L4F contains one tryptophan residue, one tyrosine residue and four phenylalanine residues. These residues exhibit fluorescence. However, phenylalanine is a weak emitter. Tyrosine and tryptophan are strong emitters but being in each other’s vicinity in the peptide molecule, they quench the fluorescence emission significantly. As a result, the shift in fluorescence of the
peptide could not be used to study quantitatively the binding between L4F and cholesterol. Hence, we chose an enzyme assay in which cholesterol acts as a substrate.

4.2.1 Design of the cholesterol binding assay

For our research, we were interested in determining the solubilization of cholesterol by L4F peptide. For this, a novel experimental approach was adopted. The low solubility of cholesterol in water, which was initially thought to be a limitation, was subsequently exploited for making a clear distinction between the cholesterol solubilized by the peptide due to binding and insoluble cholesterol that is not bound to L4F. The initial investigation was limited to comparing the solubilization of cholesterol by L4F to that by a known solubilizing agent like ethanol. Later, we developed a new method that would allow physical separation of L4F bound and hence soluble vs. unbound or insoluble cholesterol. This was achieved by centrifugation of the sample containing cholesterol and L4F peptide at 14000rpm in a microcentrifuge for 20minutes. This ensured adequate separation of soluble (bound) cholesterol from the insoluble or suspended cholesterol molecules in the sample tube. The supernatant was subsequently transferred into a new tube and was analyzed for determining the amount of cholesterol by the cholesterol detection assay. The precipitate too was subjected to quantification using the same enzyme based detection assay. It was found that the precipitate contained some cholesterol in complex with L4F. Thus the movement of cholesterol in the sample tube was traced. The general scheme for the binding assay including the detection assay using Amplex® Red enzyme kit is depicted in figure 6.
4.2.2 Principle of the cholesterol detection assay

The detection assay is based on an enzyme coupled reaction that can detect both free cholesterol as well as cholesteryl esters. Cholesteryl esters are hydrolyzed by cholesterol esterase into free cholesterol. The cholesterol is then oxidized by cholesterol oxidase to yield H$_2$O$_2$ and the corresponding ketone product. The H$_2$O$_2$ is then detected using 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex® Red reagent), a highly sensitive and stable probe for H$_2$O$_2$ [48]. Amplex® Red reagent, in the presence of horseradish peroxidase (HRP), reacts with H$_2$O$_2$ with a 1:1 stoichiometry to produce highly fluorescent...
Resorufin. Resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively [38, 48].

From our initial experiments, it was realized that the binding of cholesterol to L4F was time dependent. This was explored further to determine the time course of binding. The enzyme reaction for the assay was allowed to run for over a 12 hour duration and samples were withdrawn at specific time points for analysis. The binding appeared to be almost instantaneous with the complex falling apart gradually over time.

Thus, making use of the insolubility of cholesterol in water and centrifugation to separate L4F bound cholesterol from unbound cholesterol; we were able to gain some insights into the cholesterol binding activity of L4F.
4.2.3 Cholesterol Standard Curve

In order to determine the concentration of cholesterol based on the fluorescence reading, a standard cholesterol curve was plotted. The assay was initially standardized using the cholesterol sample included in the assay kit. With more experimentation, it was realized that a much better curve could be obtained if the cholesterol sample from the kit was replaced by laboratory grade cholesterol which was the same one used for studying interaction between L4F and cholesterol. This also helped in reducing the variability possibly produced by using different kinds of cholesterol.

The solubility of cholesterol was a highly limiting factor during experimentation. The water solubility of cholesterol is 0.02mg/L[49]. However, its solubility in ethanol was found to be 2.4mg/ml. Thus a cholesterol stock solution was made in ethanol. From this stock solution, solutions of cholesterol ranging from 0 to 30µM were prepared by diluting with distilled water. The assay protocol was followed exactly as stated in section 3.2.1.4 and a standard curve was obtained.
The equation of line was obtained from the standard curve. The $R^2$ value was found to be 0.9975 which meant that we could use the equation of line with high confidence. This also meant that the assay was standardized for use within this range of concentrations. Each assay performed later with the different samples consisted of the plotting of a standard curve and an equation of line. The graph in figure 4 is a representative of one such standard curve.

4.2.4 Cholesterol binding assay- Preliminary investigations

Since there was very little literature on interaction of cholesterol with L4F in solution state, we were interested in finding out what kind of effect the presence of peptide has on a solution of cholesterol. Cholesterol being sparingly soluble in water, the stock solutions and samples had to be made in ethanol. However, in order to study the interaction or solubilization effect of L4F, we could not have ethanol in the cholesterol solution. Thus, we designed an experiment where the ethanol was evaporated from the cholesterol.
solution using a rotatory evaporator maintained at 40°C. This temperature does not affect cholesterol physically since the melting point of cholesterol is 148-150°C[49]. 1ml of 100μM cholesterol in ethanol was added to separate microcentrifuge tubes and subjected to evaporation. Once all the ethanol was evaporated, the tubes were divided into two sets of samples. Since we did not have a reference for assessing or comparing the solubilization of cholesterol by L4F to, we decided to compare it with the solubilization of L4F brought about by pure ethanol. Thus, for preliminary examination, we added increasing amounts of ethanol (table 8) and to another set a solution of varying concentrations of L4F (table 9).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume of ethanol added to residual cholesterol (µL)</th>
<th>Volume of distilled water added (µL)</th>
<th>Final concentration of ethanol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>E2</td>
<td>100</td>
<td>900</td>
<td>10</td>
</tr>
<tr>
<td>E3</td>
<td>200</td>
<td>800</td>
<td>20</td>
</tr>
<tr>
<td>E4</td>
<td>300</td>
<td>700</td>
<td>30</td>
</tr>
<tr>
<td>E5</td>
<td>400</td>
<td>600</td>
<td>40</td>
</tr>
<tr>
<td>E6</td>
<td>500</td>
<td>500</td>
<td>50</td>
</tr>
<tr>
<td>E7</td>
<td>600</td>
<td>400</td>
<td>60</td>
</tr>
<tr>
<td>E8</td>
<td>700</td>
<td>300</td>
<td>70</td>
</tr>
<tr>
<td>E9</td>
<td>800</td>
<td>200</td>
<td>80</td>
</tr>
<tr>
<td>E10</td>
<td>900</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>E11</td>
<td>1000</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 9 Sample preparation for samples containing ethanol
Sample & Volume of 100μM L4F added to residual cholesterol (μL) & Volume of distilled water added (μL) & Final concentration of L4F (μM) 

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume of 100μM L4F added to residual cholesterol (μL)</th>
<th>Volume of distilled water added (μL)</th>
<th>Final concentration of L4F (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>P2</td>
<td>200</td>
<td>800</td>
<td>20</td>
</tr>
<tr>
<td>P3</td>
<td>400</td>
<td>600</td>
<td>40</td>
</tr>
<tr>
<td>P4</td>
<td>600</td>
<td>400</td>
<td>60</td>
</tr>
<tr>
<td>P5</td>
<td>800</td>
<td>200</td>
<td>80</td>
</tr>
<tr>
<td>P6</td>
<td>1000</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 10 Sample preparation for samples containing L4F

All samples were incubated for 30 minutes at room temperature to allow interaction between peptide and cholesterol. At the end of the 30 minutes, the fluorescence assay was performed by withdrawing 50μL of sample from each of the tubes to a fresh new tube. The protocol was followed as described in the methods section 3.2.1.4. Following were the curves obtained from the fluorescence data.
Figure 9 Comparing solubilization of cholesterol by Ethanol and L4F

From figure 9, it could be seen that the solubilizing power of ethanol was much higher than that of the peptide L4F. However, in case of ethanol, the concentration of cholesterol that could be detected was very little (less than 10µM) up to a concentration of 40% ethanol. It took more than 40% ethanol in the solution to bring about a solubilization of cholesterol. Over 40%, the amount of cholesterol solubilized increased in a fairly concentration dependent manner. For the peptide L4F, the amount of cholesterol solubilized and detected by the assay was well below 10µM. Even when the concentration of L4F was a 100µM, the concentration of cholesterol detected was only 9.3µM.

The $K_D$ of binding of L4F to cholesterol was estimated to be 11.6nM by Van Lenten *et al* using surface plasmon resonance (SPR). This indicated that the affinity of binding was
quite high[23]. Although this affinity of binding was determined when the peptide was immobilized and not in solution state, there was still not much reason to believe that the low detection of cholesterol in the assay was due to low or no binding. Several factors could be responsible for this apparent low binding or solubilization of cholesterol by L4F. Upon analyzing, we narrowed it down to the following few factors that could affect the binding of cholesterol to L4F.

1. Cholesterol was in a pellet/ solid form. The peptide could be incapable of acting upon cholesterol in solid state and pull it out of the pellet to solubilize it. This was made even more difficult considering the surrounding solvent was water and cholesterol is known to be hydrophobic. Thus unless L4F has a very high solubilizing power, cholesterol extracted out of the pellet would be fairly low.

2. For binding to occur, pH could play an important role. In this experiment, we used distilled water as a solvent without any buffers. Thus the pH was not controlled in any way.

3. Binding between cholesterol and L4F is time dependent and as a result, there was no observable increase in the amount of cholesterol bound/ solubilized with increasing peptide concentration.

4. The activity of enzymes in the assay could be inhibited or affected by the presence of L4F in some way.

We first wanted to address the issue of whether the peptide was interfering in the functioning of the enzymes cholesterol oxidase and esterase in the assay. If the peptide had any such effect, we would be unable to use the assay kit for assessing the binding any further. The easiest way of doing this was to design an experiment where the cholesterol
was in solution, available for detection and then adding increasing amounts of peptide to check if the fluorescence decreased instead of increasing. Since ethanol had shown to affect the solubility of cholesterol in the earlier experiment, we decided to use a relatively inert solvent for carrying out this experiment. Thus, we decided to use DMSO since cholesterol is completely soluble in DMSO.

Before beginning the experiment, we wanted to make sure that DMSO itself did not block the enzyme used in the assay to detect cholesterol. For this reason, we first plotted a standard curve using cholesterol in DMSO and equation of line obtained from this equation was used for further calculations. The standard curve with cholesterol dissolved in DMSO is as follows.

![Standard curve for Cholesterol in DMSO](image)

**Figure 10 Standard curve for Cholesterol in DMSO**

Since it was clear that DMSO did not affect the enzyme (detection) assay, the actual experiment with L4F was carried out. The samples consisted of a fixed amount of
cholesterol in DMSO, HEPES buffer at pH 7.4 to maintain binding conditions and increasing amount of L4F as shown in table 11

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume of 100µM cholesterol in DMSO stock solution added (µL)</th>
<th>Volume of DMSO added (µL)</th>
<th>Volume of 100µM stock of L4F in HEPES added (µL)</th>
<th>Volume of HEPES added (µL)</th>
<th>Final concentration of cholesterol (µM)</th>
<th>Final concentration of L4F (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>40</td>
<td>0</td>
<td>50</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>40</td>
<td>2</td>
<td>48</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>40</td>
<td>4</td>
<td>46</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>40</td>
<td>6</td>
<td>44</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>40</td>
<td>15</td>
<td>35</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>40</td>
<td>20</td>
<td>30</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 11 Sample preparation for DMSO-cholesterol control experiment

The samples were incubated for 30mins and the protocol for the assay was followed exactly as mentioned in the section 3.2.1.4. The results obtained are represented in the following graph.
The graph in figure 11 shows that the amount of cholesterol that could be detected without L4F added to the sample was 10.9 µM, close to the total amount of cholesterol in the sample. The total cholesterol detected when L4F was present in the sample was lower than 10 µM in all cases. However, the amount detected did not decrease with increasing concentration of L4F. This indicated that the enzyme activity was not inhibited by the increasing amount of L4F in the sample. Hence, we ruled out the possibility of the peptide inhibiting the functioning of the enzyme.

4.2.5 Optimizing the assay

To address the remaining potential causes of the apparent low binding, we designed a new experiment. Since the binding of L4F to cholesterol was detected successfully by Van Lenten et al, we decided to mimic the conditions used in their SPR experiment for allowing binding. According to those conditions, the solution of cholesterol or lipid added

<table>
<thead>
<tr>
<th>DMSO added</th>
<th>Ethanol added</th>
<th>HEPES added</th>
<th>Incubation</th>
<th>Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>30 mins</td>
<td>No</td>
</tr>
</tbody>
</table>

**Figure 11 Effect of peptide on cholesterol in DMSO**
contained up to 2% alcohol during its binding to the peptide. To maintain the pH, HEPES buffer at 7.4 pH was used[23]. Thus, we decided to add 2% v/v ethanol in the sample of cholesterol after the evaporation of solvent ethanol was carried out as described earlier.

To control the pH, the stock solution of L4F was now made in HEPES instead of distilled water.

Table 12 shows the scheme for assay indicating the amounts of ethanol, L4F and HEPES added to the solid cholesterol after solvent evaporation as well as the concentration of L4F in each sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume of ethanol added (µL)</th>
<th>Volume of 100µM stock of L4F in HEPES added (µL)</th>
<th>Volume of HEPES added (µL)</th>
<th>Final concentration of L4F (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>20</td>
<td>78</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>40</td>
<td>58</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>60</td>
<td>38</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>80</td>
<td>18</td>
<td>80</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table 12 Sample preparation with ethanol and HEPES**

After obtaining the results from our preliminary investigation, we incorporated the centrifugation step which formed the basis of our binding assay and was essential to distinguish between L4F solubilized (bound) cholesterol from the insoluble (unbound) cholesterol in the samples. The samples were now centrifuged at 14000rpm for 5 minutes before withdrawing sample solution for the assay. The idea behind this centrifugation step was that we wanted to find out if L4F increased the amount of cholesterol in solution as against the samples that did not contain the peptide. In order to separate the bound and
hence soluble cholesterol from the suspended or solid cholesterol in the tube, we decided to centrifuge the tubes before withdrawing the supernatant for the assay. Thus, at the end of the 30 minutes incubation and 5 minutes centrifugation, 50 µL supernatant was withdrawn from each sample tube and transferred to a fresh new tube. Assay protocol was followed as described in section 3.2.1.4. Following were the results obtained from the assay.

![Solubilization of cholesterol at various concentration of L4F](image)

From the graph in figure 12 we can see that the amount of cholesterol in the supernatant increases with the increase in the concentration of L4F added to the sample. This was indicative of binding and solubilization of cholesterol by L4F. However, the cholesterol that could be detected in the supernatant was only 25.7 µM even when the concentration of L4F is 100 µM. This led us to the idea that some cholesterol-L4F complex could have

<table>
<thead>
<tr>
<th>Ethanol added</th>
<th>HEPES added</th>
<th>Incubation</th>
<th>Centrifugation</th>
<th>Analysis of supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>Yes</td>
<td>30mins</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
been precipitated during centrifugation. This could explain the low concentration of cholesterol in the supernatant. Hence we needed to analyze both the supernatant and pellet or precipitate and not just the supernatant. In short, we needed to track the partitioning of cholesterol in the sample tube.

It was observed that after centrifugation, a small pellet formed at the bottom of the microcentrifuge tube in the samples that contained L4F peptide whereas a very small and diffuse precipitate was observed in the sample without peptide. Also, the relative size of this pellet increased as the amount of L4F added to the sample increased. Thus, we believed that this pellet contained the cholesterol bound to the peptide. It was now important to determine whether the cholesterol in pellet was available for detection. In order to achieve this and to successfully determine quantitatively the amount of cholesterol in both pellet and supernatant, we designed a new assay scheme. The first step was to reduce the total reaction volume to the volume that is needed for the assay which is 50µL. Thus, instead of having a reaction volume of 100µL, we reduced it to 60µL. It was also realized that the order in which the reagents were added to the tube made a difference in the size of the pellet observed after centrifugation. The pellet was of a considerable size when L4F was added to the cholesterol before diluting it with HEPES buffer. The pellet was small and somewhat diffuse when L4F was added to the diluted sample of cholesterol containing HEPES buffer. This could be due to the ability of the peptide to bind better in a small volume rather than having to bind in a diluted, larger volume. Hence it was made sure that the L4F was added to the cholesterol and then diluted with HEPES buffer. Samples were prepared as follows. All samples were made in triplicate.
The supernatant was analyzed as described earlier by pipetting out 50µL of the supernatant into a fresh new tube. In case of precipitate, a few additional steps needed to be carried out. After withdrawal of supernatant from the tube, the sample remaining in the tube was the pellet/precipitate along with 10µL of the left over sample solution. To this 10µL, 40µL of HEPES buffer was added to make up the volume to 50µL. This 50µL sample was used for the assay to analyze the amount of cholesterol in the precipitate. The general protocol of the assay was not altered in any way. However, it was realized that the assay buffer was more effective if used at a higher concentration. Thus, instead of using the diluted (1X) form of buffer, we used 5X buffer that was provided directly. Hence now the detergent concentration in the samples was similar to that in the enzyme assay. From the 50µL of supernatant and precipitate, 40µL was transferred to a fresh microcentrifuge tube to which 10µL of 5X buffer was added. It was this 50µL of sample that was mixed with 50µL of Amplex® Red reagent. Apart from this one change in step 2 and 3, the assay protocol was followed exactly as stated in section 3.2.1.4.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Volume of 5mM cholesterol in ethanol added (µL)</th>
<th>Volume of 100µM L4F in HEPES added (µL)</th>
<th>Volume of HEPES added (µL)</th>
<th>Final concentration of cholesterol (µM)</th>
<th>Final concentration of L4F (µM)</th>
<th>Final concentration of ethanol in sample (%)/ethanol added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>58.8</td>
<td>0</td>
<td>0</td>
<td>1.2µL</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>18</td>
<td>40.8</td>
<td>0</td>
<td>30</td>
<td>1.2µL</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>0</td>
<td>58.8</td>
<td>100</td>
<td>0</td>
<td>2%</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>7.2</td>
<td>51.6</td>
<td>100</td>
<td>12</td>
<td>2%</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>12</td>
<td>46.8</td>
<td>100</td>
<td>20</td>
<td>2%</td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>18</td>
<td>40.8</td>
<td>100</td>
<td>30</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 13 Sample preparation for analysis of supernatant and precipitate
Samples 1 and 2 were made as control samples. Sample 1 contained neither peptide nor cholesterol. It was made to confirm the background fluorescence. We also wanted to make sure that the peptide itself did not contribute in any way to the fluorescence readings of the samples. Sample 2 contained 30µM L4F. This was the maximum concentration of L4F used for any of the samples. Samples 1 and 2 were analyzed using the same method described above. The sample was divided into supernatant and precipitate as described earlier. Following were the observations for the experiment. The graph indicates the mean of all three readings for every sample point. The error bars represent standard deviation.

![Graph showing background reading for cholesterol in control samples](image)

**Figure 13 Background reading for concentration of cholesterol in control samples**

The graph in figure 13 clearly shows that the background is quite low. Its contribution to the reading of cholesterol in each sample is less than 0.01 µM. The peptide L4F too does
not contribute to the fluorescence by itself. In terms of concentration of cholesterol, its contribution to the reading is less than 0.05µM. The graph obtained for the other experiment for determining the amount of cholesterol in both precipitate and solution is as follows.

![Solubilization of cholesterol in supernatant and precipitate](image)

<table>
<thead>
<tr>
<th>Ethanol added</th>
<th>HEPES added</th>
<th>Assay buffer added</th>
<th>Incubation</th>
<th>Centrifugation</th>
<th>Analysis of supernatant</th>
<th>Analysis of precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>Yes</td>
<td>1x</td>
<td>30mins</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Figure 14 Concentration of cholesterol in supernatant and precipitate**

From the graph in figure 14, it can be seen that the cholesterol detected in both supernatant and precipitate increase in the presence of the peptide L4F. For the sample where there was no peptide present, the amount of cholesterol that could be detected was 0.81µM and 7.53µM in supernatant and precipitate respectively. However, in the samples containing L4F, the amount of cholesterol that was detected in both supernatant and precipitate increased as the concentration of L4F was increased. The maximum
cholesterol detected in supernatant was 2.72µM and that in the precipitate was 12.94µM in the sample containing 30µM of L4F.

Thus, the presence of peptide in the sample made it possible for the cholesterol to be detected by the enzymes. This led us to believe that the peptide plays a role in making the cholesterol available to the enzymes in the assay. To explain the surprising increase of cholesterol detected in the pellet, we hypothesized that the peptide acts as a carrier that binds the cholesterol but allows processing of the cholesterol by delivering it to suitable enzymes.

4.2.6 Detergent action of L4F

The data presented above indicated that L4F made the cholesterol available for the enzymes for further processing. Hence, the peptide showed detergent or surfactant like activity in solution. We were then interested in determining how L4F compared to a detergent known to be capable of solubilizing cholesterol. The assay buffer from the Amplex® Red cholesterol assay kit contained 0.5% Triton® X-100. Triton® X-100 is a well-known non-ionic surfactant or detergent. In the assay, it has been shown to aid maintaining the free cholesterol in solution[50]. As described in section 3.2.1.4, this buffer was used in all the samples before adding the Amplex® Red working solution. Thus the measured ability of the peptide to solubilize cholesterol or make it available for the enzyme was in fact due to both Triton® X-100 and L4F together. Hence we decided to make comparisons between Triton® X-100 and L4F.

The buffer provided with the assay kit contained 20ml of 0.5M potassium phosphate, 0.25M NaCl with Triton® X-100 maintained at a pH of 7.4. We made a blank buffer by
dissolving potassium phosphate and NaCl in HEPES. The pH was adjusted to 7.4. This was used for the new assay which consisted of three sample sets- one containing just the blank buffer prepared in the lab, another containing the buffer provided with the assay kit that has Triton® X-100 and third sample containing L4F with the blank buffer prepared above.

Before carrying out the assay with the peptide, we wanted to check how the measurement of cholesterol is affected by replacing the buffer from the kit by the buffer made in lab. The samples for this experiment were prepared as follows. All samples were made in triplicate.

<table>
<thead>
<tr>
<th>Step number</th>
<th>Process</th>
<th>Set 1 (Blank buffer)</th>
<th>Set 2 (Triton® X-100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Volume of 5mM cholesterol in ethanol added (µL)</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>Volume of HEPES buffer added (µL)</td>
<td>58.8</td>
<td>58.8</td>
</tr>
<tr>
<td>3</td>
<td>Centrifugation carried out for 20minutes @ 14000rpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Volume of buffer added to both supernatant and precipitate (µL)</td>
<td>10 µL of blank buffer</td>
<td>10µL of buffer with Triton® X-100</td>
</tr>
</tbody>
</table>

Table 14 Sample preparation for assessing effect of Triton® X-100

For control, samples were made with 1.2µL of pure ethanol instead of using the 5mM stock solution of cholesterol in ethanol. All the samples were centrifuged and separated into precipitate and supernatant. The rest of the assay was carried out as described in the earlier section. The readings for samples without cholesterol were same as that for blank and so were subtracted from each reading to correct for the background. Following are the results for the samples with and without Triton® X-100 buffer.
It can be seen from figure 15 that the amount of cholesterol detected in the supernatant was low as compared to that in the precipitate. Even then, it was not the same with or without Triton® X-100 in the buffer. In case of precipitate, the amount of cholesterol that can be detected in samples with Triton® X-100 is significantly greater than that in samples containing just the blank buffer. This proves that Triton® X-100 does aid in making the cholesterol available for enzymatic processing in the precipitate. However, cholesterol can still be detected in absence of Triton® X-100.

From our previous experiments, we had observed that the amount of cholesterol detected in the precipitate was always greater than that in supernatant in presence of the peptide L4F. This resembles the effect seen above with Triton® X-100. Hence, it is reasonable to
believe that the peptide L4F also has a detergent like action. Samples were prepared as follows.

<table>
<thead>
<tr>
<th>Step no.</th>
<th>Process</th>
<th>Set 1 (Blank buffer)</th>
<th>Set 2 (Triton® X-100)</th>
<th>Set 3 (L4F - 30µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Volume of 5mM cholesterol in ethanol added (µL)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>2</td>
<td>Volume of 100µM L4F in HEPES added (µL)</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>Volume of HEPES buffer added (µL)</td>
<td>58.8</td>
<td>58.8</td>
<td>40.2</td>
</tr>
<tr>
<td>4</td>
<td>Centrifugation carried out for 20minutes @ 14000rpm</td>
<td>20minutes @ 14000rpm</td>
<td>20minutes @ 14000rpm</td>
<td>20minutes @ 14000rpm</td>
</tr>
<tr>
<td>5</td>
<td>Volume of buffer added to both supernatant and precipitate (µL)</td>
<td>10 µL of blank buffer</td>
<td>10µL of buffer with Triton® X-100</td>
<td>10 µL of blank buffer</td>
</tr>
</tbody>
</table>

**Table 15 Sample preparation for comparing L4F and Triton® X-100**

Following were the results obtained for comparison between the detergent action of L4F and Triton® X-100.
From the graph in figure 16, it can be observed that the amount of cholesterol solubilized by L4F is 3.46µM whereas that by Triton® X-100 is only 2.3µM. Thus L4F was comparable to Triton® X-100 in solubilizing cholesterol. The amount of cholesterol detected in the samples containing L4F in the precipitate was however lower than that detected in samples containing Triton® X-100. Triton® X-100 is a non-ionic surfactant used in the assay for the sole purpose of keeping the cholesterol in solution. Hence, it was no surprise that the reading for precipitate containing Triton® X-100 was greater than that for L4F. However, what was remarkable was that L4F by itself could make considerable amount of cholesterol available for detection in the precipitate. 9.72µM cholesterol was available for detection even when it was in the precipitate form without detergent to keep
it in solution. Thus, although not as good as Triton® X-100, L4F shows some considerable surfactant/detergent action.

4.2.7 Time course of binding

From a few experiments carried out earlier, the process of binding seemed to be time dependent. The data from previous experiments showed higher readings for samples incubated for 3 hours or less as compared to those incubated overnight. The literature available so far does not illustrate the time involved in binding of L4F to cholesterol. It also does not indicate if the bound complex is stable over a period of time. Hence, to gain understanding of the process of binding over a period of time, we decided to subject the samples to multiple measurements at fixed intervals. It was essentially the same experiment where L4F and cholesterol binding was measured using fluorescence enzyme assay by making measurements at multiple time points. This was possible due to the fact that the enzyme assay was a continuous one and not an end point assay. Thus, the enzyme reaction was not quenched and was allowed to run for a long period of time.

We decided to determine the binding at the following four time points- 0 hour, 0.5 hours, 3 hours and 12 hours. In order to determine whether L4F and cholesterol have any instantaneous interaction, we decided to subject the sample to the assay measurements as soon as the sample was prepared. This was called time 0 (zero). Another sample was allowed to incubate for a short period of time- 30minutes and then subjected to the assay. The third time point selected was 3 hours. This was to find out if the amount of L4F bound cholesterol increased over the 3 hours of incubation. Since we were interested in finding out how stable the complex is, we made another measurement at 12hours. This
would illustrate if the complex stayed or fell apart over the period of 12 hours. The samples were prepared as follows.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume of 5 mM cholesterol in ethanol stock added (µL)</th>
<th>Volume of pure ethanol added (µL)</th>
<th>Volume of 100 µM L4F in HEPES added (µL)</th>
<th>Volume of HEPES buffer added (µL)</th>
<th>Final concentration of cholesterol (µM)</th>
<th>Final concentration of L4F (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.2</td>
<td>0</td>
<td>58.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
<td>58.8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>0</td>
<td>7.2</td>
<td>51.6</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
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<td>1.2</td>
<td>0</td>
<td>12</td>
<td>46.8</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>0</td>
<td>18</td>
<td>40.2</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 16 Sample preparation for time course experiment

Sample 1 was made as a control sample for background correction. Sample 2-5 contain increasing amounts of peptide L4F. Four sets of samples were made containing each of the samples shown above in the table. Each set of samples was incubated for a different period of time. Set one was not allowed to incubate at all or was incubated for 0 minutes. Set two was incubated for 0.5 hours. Set three was allowed to incubate for 3 hours and set four was incubated for 12 hours at 37°C in a water bath. At the end of the incubation period for each of the sample sets, they were subjected to centrifugation and separation of supernatant from precipitate. To both supernatant and precipitate, blank buffer (buffer containing potassium phosphate and sodium chloride) was added and not the buffer containing Triton® X-100. The rest of the assay procedure was unaltered and steps followed were exactly as per the protocol in section 3.2.1.4. Following were the results obtained.
Figure 17 Binding at time 0

At time zero, the graph in figure 17 shows that the amount of cholesterol in the supernatant increased consistently as the amount of L4F increased. These samples were not allowed to bind at all. That is probably the reason for a decrease in the amount of cholesterol detected in the sample containing 20µM L4F. Following were the results for the samples incubated for 0.5 hours.
The graph in figure 18 shows the amount of cholesterol detected after 30 minutes/0.5 hours of incubation. It can now be seen that the amount of cholesterol detected in both precipitate and supernatant increases with the increasing amount of L4F in the sample. Also, each reading at this time point is greater than that at time zero for both precipitate and supernatant. Thus it is safe to assume that the interaction between cholesterol and peptide is quantifiable only after a short period of incubation where in the binding can occur. The results for binding at 3 hours are shown in figure 19.
Upon incubation of samples for 3 hours, the amount of cholesterol detected had increased significantly as compared to that detected at 0.5 hours. The cholesterol in supernatant was found to be 5.74 µM (at 30 µM L4F) as against 4.1 µM when the samples were incubated for just 30 minutes. The amount detected in precipitate had also increased from 11.30 µM to 14.97 µM (at 30 µM L4F) after 3 hours of incubation. Thus, this indicates that the binding is time dependent. The binding results after 12 hours of incubation are as follows.

**Figure 19 Binding at 3 hours**
The graph in figure 20 shows a decline in the amount of cholesterol detected in the sample after 12 hours of incubation. There could be several factors leading to lower reading of cholesterol like degradation of cholesterol or the enzymes in the assay. The cholesterol could have degraded but that would happen more or less to the same extent in all the tubes and could be ignored as background. However, if the enzyme would have degraded, there would be no fluorescence at all and all the readings would have been low- which is certainly not the case. Hence we rule out all other possibilities. Also, the readings show a pattern that is dependent on the amount of L4F present in the sample. Thus, this is indicative of binding of cholesterol to L4F. According to our hypothesis, the complex is stable over some period of time after which it falls apart. This is proved by the readings at 12 hours, which resemble those at time 0 when not much binding could be detected.
An overlay of cholesterol detected at different time points at various concentrations of L4F as well as various time points is shown below.

**Figure 21** Cholesterol detected at all four time points in samples containing various concentrations of L4F
It is clear from the graphs in figure 21 and 22 that the binding of cholesterol to L4F is time dependent. It gradually increases after L4F is introduced in the sample tube. At 3 hours of incubation, the amount detected was the highest amongst all samples at all different time points. The complex however, does not seem to be stable over extended period of time and starts to fall apart. The amount of cholesterol detected in the supernatant after 12 hours of incubation is still higher than that seen in the sample at 0 minutes. This indicates that the solubilizing effect of L4F is somewhat more prominent that the detergent effect. Hence, the amount in precipitate at 12 hours is lower than that at 0 minutes.
Thus we have been able to determine the probable time line of activity of L4F in solution with cholesterol. With more experimentation, an exact half-life of the complex could be successfully determined.

4.3 Expression of isotopically labeled peptide

Nuclear Magnetic Resonance is a powerful tool to study interactions at a structural level. The technique of hydrogen deuterium exchange using NMR is especially well suited to study complex molecules like peptides and proteins and their interactions with other molecules. We were interested in studying the structural mechanism of the interaction between cholesterol and L4F peptide using Nuclear Magnetic Resonance (NMR) technique. For this purpose, the peptide needed to be labeled isotopically. The method used for labeling the peptide was by expressing it in bacterial cells grown in enriched medium. Several steps were involved in the process of expression. They are as described below.

4.3.1 Vector selection

Selecting an appropriate vector or expression system was the first step towards expressing an isotopically labeled peptide. Theoretically, the gene construct for the peptide could be inserted into a number of suitable vectors. However, several factors are involved in choosing the vector. The anatomy of a vector is as shown in the diagram below.
Figure 23 Anatomy of a vector expression system

The vector system for expression will generally have all the above characteristic sites encoded within itself. The selectable marker is usually the antibiotic resistance site. The gene confers resistance to antibiotics so that cells can be grown in a medium containing the antibiotic to selectively isolate the plasmids resistant to that antibiotic. The origin of replication is the site at which the replication is originated in the DNA or chromosome. The F1 origin of replication is useful for making copies of single strand of anything when placed in the correct orientation. The repressor site is responsible for inhibiting the transcription by binding to certain specific segments of the DNA. The promoter region aids in initiation of transcription. It is located upstream to a coding region. The ribosomal binding site is located at the 5’ region of the untranslated mRNA transcript and helps in regulating protein synthesis based on its sequence and structure. Multiple cloning site,
also known as the polylinker, is the region that contains several restriction enzyme recognition sites close to each other. This is very useful in cloning or inserting a piece of foreign DNA without affecting other essential plasmid functions.

The vector selected for expression of L4F was GEV 1. Marius Clore et al designed this vector specifically for obtaining high yield of isotopically labeled peptides for use in NMR studies. It consists of the immunoglobulin-binding domain of streptococcal protein G (GB 1 domain) that is linked to the N-terminus of the desired protein/peptide[39]. The advantage of using GEV 1 is that the fusion product or fusion peptide is soluble and easy to purify. The vector map for GEV1 is as follows.

![Vector map for GEV 1 including restriction sites- figure modified from the original figure by Jeffrey R. Huth[39]](image)

Figure 24 Vector map for GEV 1 including restriction sites- figure modified from the original figure by Jeffrey R. Huth[39]
The vector map of GEV1 shows the GB 1 domain upstream of the GEV1 linker that includes restriction enzyme Nhe I and Xho I recognition sites followed by the His tag. Thus the construct for the peptide can be inserted using two different restriction enzymes—Nhe I and Xho I.

### 4.3.2 Design of gene construct for expressing L4F

There were several factors that needed to be considered in designing the constructs for inserting into the vector. The oligos or constructs were to be annealed before inserting into the vector using PCR. Thus, we designed both sense and anti-sense strands of DNA expressing the peptide along with the PCR primers for making copies of the oligos for insertion. Since PCR was used, we needed to keep the melting temperature, annealing temperature and the GC content in mind. The oligos were designed using the process of reverse translation of amino acids. Using the primary sequence of the peptide, the amino acids were reverse translated into their three-letter codon form. Since the oligos were going to be inserted into bacterial cells, the codon usage bias in bacteria was considered while choosing codons for each of the amino acids. The GC content was decreased in order to reduce the melting temperature of the strands. The construct also had to include other regions like recognition sites for the restriction enzymes Nhe I and Xho I, overhangs for the enzymes to sit on and the start and stop codons. Also, for our peptide, we wanted to have a His tag at the N-terminal rather than the C-terminal as mentioned in the vector map. Hence the oligo design includes a codon for the His tag at the N-terminal. Thus, after careful consideration of all factors and optimization of codons, the final sequence of the oligos was as follows.
Oligo 1

5’ GTA\textcolor{Orange}{GCTAGC}CATCATCATCATCATCATATGGATTTGGTTCAAAGCGTTCTATGAC\textcolor{Yellow}{A}AA
GTG\textcolor{Orange}{GCCGGA}AAATTTCAAAAGAGGCGTTCTA\textcolor{Magenta}{ACTC}GA\textcolor{Cyan}{TAC} 3’

Oligo 2

5’ GTA\textcolor{Orange}{ACTCGAGTTT}A\textcolor{Yellow}{GAACG}CCTTTTGAATTTC\textcolor{Green}{TGCA}T\textcolor{Cyan}{TAC}\textcolor{Magenta}{GCTAGC}TAC 3’

The primers designed for annealing and multiplying the templates are as follows

Primer 1/ Forward primer

5’ \textcolor{Orange}{GTA}\textcolor{Cyan}{CTCGAGTTT}A\textcolor{Yellow}{GAACGC}CCTTTTGA\textcolor{Green}{ATTTC}AC\textcolor{Cyan}{TGCA}T\textcolor{Magenta}{GCTAGC}TAC 3’

Primer 2/ Reverse primer

5’ \textcolor{Orange}{GTA}\textcolor{Cyan}{GCTAGC}CATCATCATCAT\textcolor{Yellow}{CATATGGAT} 3’

Yellow – Extra bases for the enzyme to sit
Cyan – Nhe I recognition site
Green – 6x His tag
Red – Stop codon
Magenta – Xho I recognition site

The melting and annealing temperatures were found to be 70.1°C and 66.3°C respectively. The final GC content was 42.7%.
For visualization and ease of comprehension, the oligos after alignment would look like as follows-

\[
3' \text{ CTCTTTAAGTTTCTCCGCAAGATTGCTTAG} \text{ ATG 5'} \\
5' \text{ GTA GCTAGC CATCATCATCATCATATGATTGGTTCAAAGTGCGGAGAATCTCAAGAGGCGGTTC} \\
3' \text{ GTACGATCGGTAGTGTAGTAGTAGTA} \\
5' \text{ CATCGATCGGTAGTGTAGTAGTAGTA GTACCTAACCAGTTTTGCCGAAAGATACTGGTTTACCGCCTTTTAAGTTTCTCCGCAAGATTGCTTAG} \text{ ATG 5'} \\
5' \text{ CATCGATCGGTAGTGTAGTAGTAGTA GTACCTAACCAGTTTTGCCGAAAGATACTGGTTTACCGCCTTTTAAGTTTCTCCGCAAGATTGCTTAG} \text{ ATG 5'}
\]

The PCR was run as per the protocol in section 3.2.2.1. The PCR product was stored in the freezer at -20°C until further use.

4.3.3 Restriction digestion and ligation of the vector and insert

The vector GEV1 which was purchased from Addgene was processed as per the protocol described above in section 3.2.2.3 and 3.2.2.4. In order to insert and then ligate the oligo or construct into the vector, we must first cut both the oligo and the vector to generate sticky ends. Sticky ends are regions of DNA remaining after the strands are cut by restriction enzymes. The enzymes cut in such a way that it leaves overhangs of DNA for complementarity. Thus, in the oligo or insert, the strands cut with Nhe I and Xho I will orient themselves in such a way that they are complementary to the “sticky ends” generated in the vector by the same enzymes. The isolated vector and the oligos were subjected to restriction digestion using the enzymes Nhe I and Xho I. The protocol was followed exactly as described in section 3.2.2.5. At the end of the digestion, the samples were subjected to gel electrophoresis to confirm bands of the digested vector and oligo.
However, we realized that we had several problems in this process. The first problem was that although the literature indicated that the enzymes Nhe I and Xho I work for cutting the plasmid and inserting the oligo in, we had difficulty obtaining plasmid once it was subjected to restriction digestion. There were either multiple bands upon digestion of the vector or no bands at all. We tried to investigate the cause for this and realized upon studying the restriction map of the vector that the GEV1 vector backbone also contained an extra Nhe I site. The information provided by the company was inadequate to analyze if this site on the backbone had been modified or removed when the plasmid was reconstructed to incorporate the GB1 domain and the His tag. The occasional success with digestion of the vector proved to be not very fruitful since the ligation reactions failed repeatedly. Several approaches were tried to identify and fix the problems with restriction digestion and ligation reactions.

1. To rule out the possibility of the enzymes having become inactive due to improper storage, fresh new enzymes were ordered and tried.
2. The concentrations of enzymes and the time of incubation for these reactions were altered to find out if time affected the process of digestion in any way.
3. The plasmid was cultured several times from different stab cultures to analyze if the plasmid isolated was indeed the vector GEV1. Antibiotic concentrations in the cultures were also varied to test selectivity of the vector.
4. Partial digestion using ethidium bromide was carried out in order to determine if the number of digestion products could be manipulated and if the desired fragment could be obtained not allowing the plasmid to digest entirely.
Despite these attempts, the ligation reactions failed to give the desired product. Efforts to express the peptide L4F using the construct and a suitable vector will be continued in the future by other lab members.
Chapter 5

Conclusion

The need for novel drug molecules for the prevention and treatment of cardiovascular diseases is ever increasing. With this increasing demand for a remedy, every effort made to discover, develop or analyze a potential drug molecule takes us one step closer to finding the cure. L4F is one such molecule with tremendous potential. It can not only treat but also prevent several conditions that lead to or aid in the development of cardiovascular diseases. Thus is can be used for both prophylaxis and treatment of lipid related cardiovascular disorders.

Through our research, we determined that L4F is capable of binding cholesterol in solution state. It has a solubilizing effect on cholesterol. This is significant since this indicates that it is capable of dissolving or solubilizing cholesterol deposited in arteries-as is the case in atherosclerosis. We also established that L4F has a detergent like action on cholesterol. This effect was compared to the suspending or solubilizing effect seen with Triton X-100 which is a known non-ionic surfactant. L4F was not as effective in removing or resuspending cholesterol precipitated from solutions. This lower activity of L4F compared to Triton X-100 is in fact a desired quality making L4F better suited for being developed into a drug. The reason for this lies in the fact that cholesterol is an essential component of plasma membranes. If L4F were capable of solubilizing cholesterol as efficiently as the detergent Triton X-100, it would have deleterious effects on the plasma membranes making the cells leak its contents or disrupt signaling or other
functions and eventually die. Hence, L4F seems to strike the perfect balance when it comes to its capabilities of binding cholesterol.

Through time course experiments, we were also able to establish that the binding of L4F to cholesterol is time dependent. The binding seems to be negligible for detection at the instant when solution of L4F is added to a sample of cholesterol. It however increases over a period of just a couple of hours with the maximum binding at 3 hours. The L4F-cholesterol bound complex however is not very stable over longer duration. This was indicated by low readings of cholesterol at 12 hours of incubation. We hypothesized that the complex begins to form almost instantaneously and continues until 3 hours when it has reached its peak. After that, it gradually starts to fall apart where at 12 hours; we have almost none that can be detected. Thus, in our opinion, the peptide L4F affords as an effective shuttling system for cholesterol. The half-life for L4F-cholesterol complex can be considered to be appropriate, if it were to be developed into a drug molecule.

These findings will certainly add to the existing knowledge of L4F and the efforts to better understand the wide range of effects of this peptide. This study takes us a step ahead in our ultimate goal of analyzing the effect of structure on the activities of L4F. Our lab will continue to contribute to this research on L4F by undertaking structural studies by Hydrogen-Deuterium Exchange using NMR or MS.
References


42. QIAgen, QIAquick® Gel Extraction Kit Protocol. 2008.


49. *Material Safety Data Sheet Cholesterol MSDS*.