IN VITRO CHARACTERIZATION OF SIMVASTATIN LOADED MICROSPHERES IN THE POLYRING DEVICE

A Thesis

Presented to

The Graduate Faculty of The University of Akron

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

Anusha Vishwanathan

May, 2008
IN VITRO CHARACTERIZATION OF SIMVASTATIN LOADED MICROSPHERES
IN THE POLYRING DEVICE

Anusha Vishwanathan

Thesis

Approved:                     Accepted:

Advisor                      Department Chair
Dr. Stephanie T. Lopina      Dr. Daniel B. Sheffer

Committee Member             Dean of the College
Dr. Daniel B. Sheffer        Dr. George K. Haritos

Committee Member             Dean of the Graduate School
Dr. Steven P. Schmidt        Dr. George R. Newkome

Committee Member             Date
Ms. Michelle Evancho-Chapman
ABSTRACT

Intimal hyperplasia is the most common mechanism of failure for interventional and revascularization procedures like coronary artery bypass grafts (CABG), peripheral artery bypass surgery and dialysis access grafts. It is characterized by the thickening of intima within the artery wall. The reason for the thickening is due to the proliferation of medial vascular smooth muscle cells in the intima of the vessel wall. In a previous study, a novel targeted drug delivery system named the “PolyRing” was devised, using the drug cyclosporin A (CyA).

The objective of our present research was to modify the previously established PolyRing system using the drug simvastatin, a hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor. The research hypotheses were that the loading of simvastatin in the PLGA microspheres could be optimized, and that there was controlled local release of the drug from the PolyRing device over a period of time. In this system, simvastatin was encapsulated within PLGA microspheres, which in turn were entrenched in a PEG block as a polymeric vascular wrap. The microspheres were prepared by an oil-in-water (o/w) emulsion technique. In order to achieve the maximum load, a total of eight runs with various formulation parameters including drug to polymer ratio, surfactant concentration, emulsification time and aqueous volume were conducted. Characterization
of the microspheres was then completed to examine the surface morphology, particle size
distribution, drug loading, encapsulation efficiency and the device’s in vitro release
behavior. For this study, the null hypothesis was that there were no significant effects of
the formulation parameters on encapsulation efficiency.

Approximately 100µm size diameter spherical, smooth microspheres were obtained
with no appreciable drug loss on the surface. The highest drug loading was observed to
be 81.5 ± 6.3 µg drug/mg of microspheres. Only the drug to polymer ratio had significant
effect on the encapsulation efficiency with the highest simvastatin encapsulated within
the microspheres being 81 ± 9%. In vitro release behavior could not be determined due to
the extremely low levels of drug detection in the release medium. Results from a series of
experiments performed suggested that there was detection of simvastatin acid, an active
metabolite of simvastatin. Alternate means for a suitable detection method have been
proposed as future work.
DEDICATION

I would like to dedicate this thesis to my grandfather and father.
ACKNOWLEDGEMENTS

I am deeply grateful and indebted for the total support and encouragement Dr Lopina has given me. Dr Steven Schmidt and Michelle Evancho-Chapman have my heartfelt appreciation and regard. Without the three above-mentioned distinguished people’s guidance, I would not have come so far. My sincere gratitude to Dr Sheffer and Dr Yun for agreeing to be in my committee and also equally for their understanding and help. A singular mention of Dr Tracy Richey of Oakwood Labs needs to be made. She helped me several times for the particle size data in spite of her busy schedule. Donna, what can I say, we had good times and I am very sorry that it had not lasted much longer. I am much in debt to Dr Ping Wang of the Chemical Department who allowed me the generous use of his HPLC equipment.

A number of people have helped me in this research process. First and foremost is Deenu Kanjickal, whose project I am following. The others include Bilal, Archana, Ravi, Debanjan, Parth, Aadithya, Pallavi, and many more. I thank all of them from the bottom of my heart. A very special mention goes to Dr Rittgers for his unfailing words of encouragement and our almost regular roadside meetings. I always look forward for it Sir. To the Health and Safety Department team, thank you for the continuous support and the encouragement.

Finally, my family who have been my backbone throughout the whole process. There cannot be enough words in the world to express my feelings. Thank you.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>.................................................................</th>
<th>x</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>.........................................................................................</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTER

### I. INTRODUCTION

1.1 Objectives ................................................................................................................................................. 4

1.2 Hypothesis....................................................................................................................................................... 4

### II. LITERATURE REVIEW

2.1 Pathology ..................................................................................................................................................... 6

2.2 Perivascular Controlled Drug Delivery System-PolyRing............................................................. 10

2.2.1 Mechanisms of Perivascular Drug Transport ....................................................................................... 12

2.3 Cell Cycle of the Smooth Muscle Cell..................................................................................................... 17

2.4 Statins.......................................................................................................................................................... 19

2.4.1 Inhibition Mechanism of Statin............................................................................................................... 20

2.4.2 Structure of Statin...................................................................................................................................... 23

2.4.3 Pharmacokinetic Properties of Statins.................................................................................................. 25

2.4.3.1 Absorption............................................................................................................................................ 25
2.4.3.2 Distribution

2.4.3.3 Metabolism

2.4.3.1 Drug Interactions

2.4.3.4 Elimination

2.4.4 Safety of Statins

2.4.5 Pleiotropic Effects of Statins

2.4.5.1 Endothelial Function

2.4.5.2 Platelet Activity and Plaque Stability

2.4.5.3 Vascular Inflammation

2.4.5.4 Smooth Muscle Cell Proliferation

2.5 Current State of Art Device-Vascular Wrap

III. MATERIALS AND METHODS

3.1 Materials

3.2 Device Fabrication

3.2.1 Preparation of PLGA Microspheres

3.2.2 Preparation of PolyRing Device

3.2.3 Device Sterilization

3.3 Characterization of Microspheres

3.3.1 Microsphere Surface Morphology

3.3.2 Particle Size Analysis

3.3.2.1 Optical Microscope Analysis

3.3.2.2 Light Scattering Analysis
3.4 Drug Loading Studies........................................................................................................ 45

3.4.1 Reverse Phase High Pressure Liquid Chromatography.................................. 46

3.5 In Vitro Release Studies............................................................................................... 47

IV. RESULTS AND DISCUSSION..................................................................................... 48

4.1 Microsphere Characterization..................................................................................... 48

4.2 Optical Microscope Analysis of Blank PLGA Microspheres ..................................51

4.3 Drug Loading Analysis ..............................................................................................52

4.3.1 Particle Size Analysis using Light Scattering Method .................................. 54

4.3.2 Actual Drug Loading and Encapsulation Efficiency .................................... 56

4.4 In Vitro Drug Release studies.................................................................................... 61

V. CONCLUSIONS........................................................................................................... 67

VI. FUTURE WORK............................................................................................................. 69

REFERENCES.................................................................................................................... 71
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Different pharmacokinetics properties of statins</td>
<td>25</td>
</tr>
<tr>
<td>4.1</td>
<td>Experimental design of a $2^{4-1}$ fractional factorial design</td>
<td>53</td>
</tr>
<tr>
<td>4.2</td>
<td>Encapsulation efficiency, drug loading and mean diameter</td>
<td>57</td>
</tr>
<tr>
<td>4.3</td>
<td>Analysis of variance</td>
<td>57</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Schematic diagram of a normal arterial wall</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic illustration of the PolyRing device</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematic diagram of cross section of drug transport pathways into the arterial wall after perivascular drug delivery</td>
<td>17</td>
</tr>
<tr>
<td>2.4</td>
<td>Cell cycle of a normal smooth muscle cell</td>
<td>19</td>
</tr>
<tr>
<td>2.5</td>
<td>Pathway for cellular proliferation and statin inhibition</td>
<td>22</td>
</tr>
<tr>
<td>2.6</td>
<td>HMG-CoA reductase inhibitor Type 1 (Simvastatin), Type 2 (Fluvastatin)</td>
<td>24</td>
</tr>
<tr>
<td>2.7</td>
<td>Interconversion kinetics of simvastatin, simvastatin acid</td>
<td>27</td>
</tr>
<tr>
<td>2.8</td>
<td>Pleiotropic effects of statins</td>
<td>33</td>
</tr>
<tr>
<td>2.9</td>
<td>Schematic illustration of Vascular Wrap</td>
<td>38</td>
</tr>
<tr>
<td>2.10</td>
<td>Schematic illustration of the PolyRing treatment strategy</td>
<td>39</td>
</tr>
<tr>
<td>4.1</td>
<td>Scanning electron micrograph of blank PLGA microspheres</td>
<td>49</td>
</tr>
<tr>
<td>4.2</td>
<td>Scanning electron micrograph of simvastatin loaded PLGA microspheres</td>
<td>50</td>
</tr>
<tr>
<td>4.3</td>
<td>Scanning electron micrograph of rhodamine loaded polyanhydride microspheres</td>
<td>50</td>
</tr>
<tr>
<td>4.4</td>
<td>Effect of various impeller speeds on blank microspheres diameter by optical microscopy</td>
<td>51</td>
</tr>
</tbody>
</table>
4.5  Graph of particle size distribution of simvastatin loaded PLGA microspheres of Run 4 as optimized from the experimental design table 4.2…55
4.6  Particle size distribution of eight runs as observed in the experimental Design table 4.2……………………………………………………………………………55
4.7  Graph of the drug loaded microspheres of eight runs. SET 1 comprising of 10% drug loading, SET 2 comprising of 20% drug loading………………………….59
4.8  Drug loading distribution of eight runs as observed in the Table 4.2………59
4.9  Graph of the encapsulation efficiency of eight runs. SET 1 comprising of 10% drug loading, SET 2 comprising of 20% loading…………………………..61
4.10 Picture of swelled and dry PolyRings………………………………………………63
4.11 Chromatograph for simvastatin loaded microspheres of Run 4 for the encapsulation efficiency study…………………………………………………………64
4.12 Chromatograph of simvastatin loaded microspheres in the PolyRing for the release study ………………………………………………………………65
CHAPTER I
INTRODUCTION

According to recent estimates from the American Heart Association, the number of cardiovascular operations done in United States from 1979 to 2002 has increased significantly by 470% [1]. Out of these, interventional procedures such as percutaneous transluminal coronary angioplasty (PTCA) and cardiac revascularization, commonly known as the coronary artery bypass graft surgery (CABG) [1] comprised the highest proportion of surgeries along with autogenous and synthetic dialysis access grafts [2,3]. It was identified that 20-30 % of patients diagnosed with peripheral vascular disease (PVD), which affects the lower extremities, undergo interventional procedures including peripheral artery bypass as well as percutaneous revascularizations [4]

In all the procedures mentioned, the surgeries were beset by recurrent stenosis and thrombosis on a long term basis even though reconstruction was accomplished immediately after intervention. Consequently, these procedures led to a higher frequency of operations for maintaining vessel patency. The most common basis for failure for these procedures was intimal hyperplasia (IH). This led to the closing or narrowing of an artery, which prevented the blood flow which normally occurs after the interventional
measures have been done. The structural changes underlying intimal hyperplasia included accumulation of new tissue within the artery wall and shrinkage of the artery wall at the site of surgery [5].

Intimal hyperplasia (IH) was the process by which the vascular smooth muscle cell (VSMC) population increases within the innermost layer of the arterial wall. The basic causes of IH were pathologically aggravated by injury, inflammation and increased mean wall stress [6]. Revascularization procedures could induce IH specifically by graft compliance mismatch, flow disturbances, foreign body reactions, activation of platelets and surgical trauma. To inhibit the proliferation, a variety of treatments have been developed including drug eluting stents and brachytherapy, but their efficacy was not high due to in-stent restenosis, target vessel revascularization (TVR), late thrombosis and total occlusion [7,8]

In a previous study at the Summa Health System by Kanjickal et al [9,10], the PolyRing device was developed, for the treatment of vascular IH. In this novel targeted drug delivery system, the drug cyclosporine A (CyA) was encapsulated within the poly (DL lactide-co-glycolide) (PLGA) microspheres which were, in turn, embedded in a poly ethylene glycol (PEG) block as a polymeric vascular wrap. This polymeric wrap was termed”PolyRing”, for which a patent has been filed (20050245905). CyA’s pathway of action for the inhibition of vascular smooth muscle cells is through the immune system [11]. Therefore statin, another potent agent with antiproliferative properties, has been suggested for the inhibition of IH. Statins have a more direct route for the inhibition of smooth muscle cells.
In large scale clinical trials, 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, referred to as statin, was found to decrease the morbidity and mortality in coronary artery disease (CAD) including that of vascular proliferative diseases [12,13]. Statin was already on the market and was consumed orally in the form of pills. It reduced the formation of cholesterol in the liver and other tissues by the up regulation of the low density lipoprotein (LDL) receptors in the liver. This resulted in the endocytosis of LDL and, consequently, a decrease in LDL levels [14]. These cholesterol lowering drugs have been demonstrated to exert significant antiproliferative, anti inflammatory effects notably benefiting cardiovascular events other than the lipid lowering action [15,16]. The effect of statin in VSMC proliferation was profound in that it directly inhibits the growth of the smooth muscle cells [17], thereby preventing the formation of IH. As a result, statins were ideal candidates to be used in the PolyRing device as an alternative to CyA for a localized controlled drug delivery system. Though statins have been associated with adverse side effects including hepatic toxicity and myopathy including rhabdomyolysis [18], the use of a localized controlled drug delivery system should eliminate them.
1.1 Objectives

The long term goal of the project was to develop a PolyRing device loaded with the statin drug capable of inhibiting vascular IH in invasive vascular procedures. The first step in realizing the long term goal was to modify the established system by using simvastatin instead of CyA and to characterize the drug loaded microspheres in the PolyRing device. Therefore, the objectives of the study were namely

1. To synthesize microspheres loaded with the statin drug
2. To optimize formulation parameters for obtaining the highest drug encapsulation efficiency
3. To incorporate statin loaded microspheres in a PEG hydrogel block polymer and subsequently a ring device
4. To determine the release kinetics of the device for both unsterilized and sterilized PEG hydrogel block polymer.

1.2 Hypothesis

The basis of our study was to test both individual research hypotheses and the null hypotheses as a result of the experimentation. The research hypotheses were namely,

- A measurable amount of simvastatin was loaded into the PLGA microspheres without appreciable amount discerned on the surface of the microspheres
- Simvastatin loaded microspheres were suspended in the PEG hydrogel block polymer without any significant loss of microspheres and without any appreciable degradation
- Sustained release of simvastatin over a period of 80 days from the unsterilized PolyRing device was achieved
The null hypotheses for the study were that

- The formulation parameter, drug to polymer ratio has no significant effects on drug encapsulation efficiency
- The formulation parameter, surfactant concentration has no significant effects on drug encapsulation efficiency
- The formulation parameter, emulsification time has no significant effects on drug encapsulation efficiency
- The formulation parameter, aqueous volume has no significant effects on drug encapsulation efficiency

Our overall research hypothesis was that there was controlled local release of simvastatin from a polymeric vascular wrap or namely the PolyRing device over a sufficient period of time.
CHAPTER II
LITERATURE REVIEW

2.1 Pathology

The most common basis of failure for revascularization as well as for interventional vascular procedures is intimal hyperplasia. Injury due to these procedures induces a wound healing response that causes the proliferation of smooth muscle cells in the arterial wall leading to intimal hyperplasia. The artery wall (Figure 2.1) is made of three distinct layers: tunica intima, tunica media and the tunica adventitia. The intima forms the innermost membrane, which includes a continuous endothelial monolayer on a specialized extracellular membrane, the extracellular matrix (ECM) or basement membrane. This layer consists of endothelial cells along with type IV collagen, laminin and proteoglycans. The middle layer is the media, which contains the smooth muscle cells, fibronectin and densely packed type I and type III collagen. The medial layer contributes to the thickness and strength of the artery. The outermost layer is the adventitia, which has the fibroblasts in white connective tissue and also other blood vessels.

Physiologically, proliferation of smooth muscle cells takes place during closure of the ductus arteriosus after birth, during involution of the uterus and also as a person ages due to the expansion of existing cell numbers [19]. Normally in adults, VSMC cells are
quiescent and proliferate at low frequency. However, when an inflammation (in response to injury) or wall stress (due to vein grafting or pulmonary hypertension) occurs, growth factors like the platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β1 as well as thrombin, endothelin-I and angiotensin II are activated [20].

There are several mechanisms behind the immediate and progressive release of thrombogenic, vasoactive and mitogenic factors including platelet adhesion caused by the exposure of collagen which, in turn, is based on the injury that might be either at the endothelial (intima level) or further at the medial layer. The release of thrombin also takes place leading to additional platelet activation. PDGF is released [20] due to platelet aggregation, which in turn stimulates fibroblasts with the help of FGF. Inflammation helps the macrophages release MMP (matrix metalloproteinases) which is essential for intima formation. This cascade effect helps in bringing about the smooth muscle cell propagation from the media to the intima layer ultimately resulting in a phenotypic change wherein there is proliferation of cells and synthesis of extracellular matrix. The proliferation and the secretion of ECM together make up the general term “intimal thickening” [21].

There have been many methods to address the problem of intimal hyperplasia including drug eluting stents and brachytherapy, but these procedures have limited success and are plagued by in-stent restenosis, total occlusion and thrombosis [7,8]. These problems lead to further surgery, which is not desirable. This gave rise to polymer based drug delivery systems, wherein elevated local levels of the drug increased the
effectiveness of the drug over a period of time, while reducing the systemic levels of the drug [22,23]

The idea behind perivascular drug delivery system is that drug, when administered perivascularly, inhibits smooth muscle cell proliferation and migration from the adventitial level. Adventitia, the outer layer of the arterial wall has been found to play an important role in neointima formation after balloon angioplasty in a swine model as discovered by Shi Y et al [24]. A study by Diaz-Flores L and Dominiguez C [25] has shown that intimal thickening can originate in cells from vasa vasorum. Many researchers have established that perivascular vehicle systems can potentially inhibit intimal hyperplasia, including Masaki et al who proved that a sustained release system for perivascular delivery of dipyridamole [26], an antiproliferative drug, could be achieved successfully. Local periadventitial delivery of tryphostin was shown to significantly inhibit intimal hyperplasia in the rat carotid injury model [27]. Edelman et al [28] have found that periadventitial controlled release of heparin inhibits smooth muscle cell formation within the blood vessels following injury to the endothelium.

The current work, the PolyRing could be capable of encapsulating a broad range of therapeutic drugs with the possibility of combination therapy. The device design is also unique in a way that made additional retention devices unnecessary thus keeping the device in close contact with the vessel adventia.
Figure 2.1: Schematic diagram of a normal arterial wall
2.2 Perivascular Controlled Drug Delivery System - PolyRing

Kanjickal et al [9,10] devised a novel localized drug delivery system for combating intimal hyperplasia, induced due to the revascularization procedures. This is achieved by potentially inhibiting the proliferation of smooth muscle cells by the drug cyclosporine A (CyA), an immunosuppressive agent. PolyRing is in the form of a ring structure, which is a polymeric device consisting of PLGA microspheres embedded in a PEG hydrogel block polymer as seen in Figure 2.2. PEG was considered excellent for biomaterial applications due to its biocompatible and non toxic nature. The PEG-PLGA block polymer in the use of controlled release system has a huge advantage of protein resistivity [29]. Hydrophilic PEG has been found to be most effective in repelling proteins within aqueous environments [29], thus preventing polymer-cell interactions in vivo. The PolyRing device has an axial slit which helps the ring to slip around the arteries as well as around vascular grafts where the anastomatic intimal hyperplasia may develop. The ring is made secure by matching the size of the ring with the diameter of the blood vessels. The drug, which is encapsulated within the PLGA microspheres, releases through the PolyRing device by diffusion and over a period of time, degradation of the polymer. The drug is transported through the perivascular space into the artery wall by various physical forces including diffusion, partitioning coefficient effect and convective forces over an extended period of time thus inhibiting the proliferation of vascular smooth muscle cells in the medial/intimal layer.
Figure 2.2: Schematic illustration of the PolyRing device [9]
The PolyRing did not need additional retention devices as it could be securely held to the wall due to the swelling of the PEG hydrogel. It is also capable of multiple drug encapsulations which could be beneficial for combination therapy. Localized CyA release is preferred to systemic delivery because the characteristics of this particular drug include nephrotoxicity in higher doses as well as low bioavailability. The method of action for CyA is inhibition through the immune system mediation rather than direct smooth muscle cell inhibition. Therefore, a complementary approach was needed in order to inhibit the smooth muscle cells more directly and this led to the study of statins which are HMG-CoA reductase inhibitors.

2.2.1 Mechanisms of Perivascular Drug Transport

Controlled release drug delivery devices provide local elevated concentrations in the targeted sites while diminishing the systemic side effects. After simvastatin is released from the device, it has to pass through the interstitial space into the adventitial tissue layer to the media, then into the intima, after which it will be carried into the lumen and systemic circulation. For the effective delivery of simvastatin at an optimum concentration for a sufficient amount of time, apart from the characteristics of the drug, the physical forces governing the transportation of the drug as well as the local tissue structures in the arterial wall need to be duly understood.

The major drug transport parameters to be considered are diffusivity, partition coefficient and to a lesser degree the convective forces involved [30,31]. Diffusion pertains to the rate of drug penetration across the pathway. The arterial wall is highly heterogeneous and each layer will have its own unique diffusivity. Hydrophobic drugs
including simvastatin, in contrast to hydrophilic drugs, are relatively insoluble and will tend to bind to hydrophobic sites in the tissues [32]. This binding slows down the movement of the drug across the arterial wall thereby localizing the drug close to the delivery site and thus increasing their release period. Diffusivity can be either in the transmural (across the elastic sheaths) or planar (parallel to the plane of elastic sheaths) direction. The diffusivity has been detected to be slightly higher along the planar direction as compared to the transmural [31]. This might be due to the fact that differences in permeability between wall layers tend to hinder the transmural mediated transport whereas along the planar region permeability differences are less due to the sameness of the layer. It has been observed that hydrophobic drug transport is supported in the lipid regions and the elastic lamina where there are more proteins, and is delayed in the non lipid regions [31].

Another key aspect is the partition coefficient which is defined as the concentration ratio of drug between two immiscible solvents or distinct phases or entities. It could be generally calculated in vitro, by concentration ratio of drug between octanol and water as octanol is capable of hydrogen bonding a correlation similar to phospholipids and proteins in biological membranes [33]. For hydrophobic drugs, the partitioning coefficient is higher as compared to hydrophilic drugs as the drug concentration in the hydrophobic solvent (namely octanol) is greater than in the hydrophilic solvent (namely water).

In vivo, the partition coefficient is defined as the concentration ratio of drug between the tissue and the blood. Hydrophobic drugs have a higher partition coefficient, as each
drug molecule repeatedly binds and disassociates with the binding sites along the arterial wall in the process of diffusing down its concentration gradient. Thus drugs when delivered through perivascular means, have a higher concentration on the adventitial/medial layer [34] as compared to the intima and the lumen. As a result of the repeated binding and disassociation along the arterial wall, transport of the drug through the tissue [35,36] is slowed down facilitating the distribution of the drug into the tissues and in consequence, prolonging tissue residence and therapeutic effect. Creel et al [37], used paclitaxel, a hydrophobic drug to study its distribution and deposition through endovascular and perivascular application. The results obtained displayed higher drug concentration levels in the intima for the endovascular application and in the adventitia for the perivascular application.

Convective forces in the vessel wall take part in a small role in drug transport. Convective forces are pressure dependent, driven by the transmural hydrostatic pressure gradient across the arterial wall, as opposed to diffusion forces which are concentration driven. Convective forces are always directed inside to out across the wall. Due to the perivascular nature of delivery, there is an opposing force between endovascular and perivascular space [38]. This higher hydrostatic pressure in the lumen might impede the flow of drug from perivascular side to the interior to some extent. But convective forces are limited, diffusion and to a greater degree the partitioning effect play a far greater role in transportation of simvastatin.

All three factors, diffusivity of drug, partitioning, subsequent binding and convective flow together affect the transportation of simvastatin across the arterial wall.
Other parameters influencing the drug transportation are the surface area of the perivascular application and drug loss during the arterial uptake of the drug. The higher the surface area of application, higher is the release of drug initially, which could potentially decrease the effective amount of the drug retained in the polymeric device, for extended release necessary for inhibition. Alternatively, smaller surface area could result in higher quantity of the drug retained for an extended release over for a longer period of time. Hence, there should be an optimum surface area for the desired release along with keeping the therapeutic efficiency in sight. Binding to non-specific sites during partitioning, loss to the systemic circulation due to diffusion and convective forces are some of the reasons for drug losses.

In all the factors mentioned above, the local surrounding tissues play an important role in the transvascular transportation of simvastatin. There are the different pathways in which the drug could be cleared from the perivascular space and subsequently deposited within the blood vessel wall. The potential routes for simvastatin losses might be due to transarterial transport with the drug entering the lumen flow and the absorption into the extraarterial capillaries [39] as shown in Figure 2.3.

The amount of simvastatin entering the lumen though could be potentially lower than the amount retained in the arterial wall through direct diffusion from the drug delivery device. This phenomenon could be attributed to the drug, binding to the hydrophobic binding sites in the vessel wall and having a higher partition coefficient as detailed above. The other means by which the drug could be cleared from the perivascular space is by absorption of drug by extraarterial capillaries. Extraarterial capillaries are defined as the
source of clearance outside of arterial wall and include the tissues surrounding the vascular wall. However, the drug absorbed could potentially reenter the arterial wall by endovascular route via systemic circulation. An approach to minimize this extraarterial capillary absorption could be to devise an impermeable wrap surrounding the artery and drug device. Lovich et al [39] used a thin Silastic sheath wrapped circumferentially around the artery and the Poloxamer gel. The ends of this sheath as well as the seam were capped off with a biocompatible seal so as to prevent leakage. Though it did not result in statistically increased local arterial concentrations, the release of drug from the gel was slowed down by restricting exposure to the interstitial
Figure 2.3: Schematic diagram of cross section of drug transport pathways into the arterial wall after perivascular drug delivery. a) Direct diffusion from polymeric device b) entry into the lumen blood flow c) extraarterial capillaries absorption of the drug d) deposition of drug through endovascular application from systemic circulation.

2.3 Cell Cycle of the Smooth Muscle Cell

Statins which are cholesterol reducing drugs, have anti proliferative effects on vascular smooth muscle cells. The cell cycle must be understood to gain better insight into the process. Different thrombogenic, vasoactive and mitogenic factors all contribute to activation of smooth muscle cells. The cycle starts from the Gap (G) O phase of the cell, which is triggered to move to the G1 phase. It passes on to the S (DNA synthesis)
phase after first passing through a restriction site R. From the DNA synthesis phase, it then goes on to mitosis (M) as shown in Figure 2.4. The restriction site R is where the retinoblastoma tumor suppressor protein (Rb) is present and transition from the G1 to S phase requires increased phosphorylation of Rb [40]. Phosphorylation of Rb leads to the release of transcription factor E2F resulting in activation of genes for DNA synthesis [41]. Common regulators of the cell cycle are present for the smooth muscle cell DNA synthesis as well as for the phosphorylation of Rb. These regulators include the cyclins, the cyclin dependent kinases (Cdk) and its inhibitors. The Cdk inhibitor consists of p16, p21waf1 p27Kip1 [41]. The role of statins in the inhibition of the smooth muscle cell proliferation and in particular with the Cdk inhibitor and effect on the phosphorylation of Rb will be discussed in length in the later section under pleiotropic effects of statins.
2.4 Statins

Statins or HMG-CoA reductase inhibitors are a group of cholesterol lowering drugs, which target the rate limiting enzyme in the cholesterol biosynthesis. It has been recently proven that apart from treatment of hypercholesterolemia and hypertriglyceridemia [42], statins are successful in coronary related developments, for example- the reduction of
post angioplasty restenosis, coronary bypass occlusions and transplant arteriosclerosis [16,43,44].

2.4.1 Inhibition Mechanism of Statin

The method of action of statin is through the mevalonate (MVA) pathway, which leads to the cholesterol synthesis as well as in the cell growth and development. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), formed from the acetyl CoA and acetoacetyl CoA, is converted to mevalonate (Figure 2.5) catalyzed by the enzyme HMG CoA reductase. Mevalonate then forms the downstream intermediates, a succession of isoprenoids that are ultimately vital for cell proliferation and cholesterol production. Isoprenoid products include geranyl geranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) [45,46]. FPP branches into squalene, which is the precursor compound to cholesterol and the prenylated proteins Ras and Rho, derived from both GGPP and FPP, is responsible for cell propagation. Ras is involved in the cellular growth through activation of mitogen activated protein kinase pathway whereas Rho is more actively involved through destabilization of the p27kip1 [47].

Statins inhibit the growth and migration of the smooth muscle cells by blocking the action of the enzyme HMG-CoA reductase to form mevalonate. Thus the intracellular synthesis of mevalonate along with isoprenoid derivatives is inhibited by the preferential binding of the enzyme to statin instead of HMG-CoA. For example, there is a strong affinity of HMG-CoA reductase to simvastatin drug, which is 13,000 times greater than that for HMG-CoA [48] consequently inhibiting further synthesis. However, the effects of the reversible inhibitors of HMG-CoA reductase are prevented by the addition of mevalonate and not by the addition of either squalene or cholesterol. Thus proving that
additionally, statins have non lipid effects and that there is an association of non sterol compounds to the cell growth [49].
Figure 2.5: Pathway for cellular proliferation and statin inhibition
2.4.2 Structure of Statin

There are different types of statins available on the market and still more are being added to the list. The chief among them are atorvastatin, cerivastatin, lovastatin, pravastatin and simvastatin. The structure of statins (Figure 2.6) is that they all share a HMG moiety but have structural differences to form their slightly individual characteristics.

The structure of HMG-CoA reductase enzyme differs according to the type of CoA reductase. The class I HMG-CoA reductase is classified as eukaryotic types and the class II as the prokaryotic. The one of interest is the class I species, particularly the human HMG-CoA reductase structure wherein the catalytic portion is a tetramer having individual monomers, arranged in dimers that form the active sites [50]. These statins, which include lovastatin, simvastatin, atorvastatin, cerivastatin, pravastatin and fluvastatin, are heavier and more hydrophobic than HMG-CoA. The binding of these statins produces a conformational change in the enzyme by allowing a tighter hydrophobic attachment thereby sterically preventing the substrate from binding [51]. All statins, which share a HMG moiety, bind at the active site and are primarily ionic or polar. On the other hand, there are structural differences in the various statins, such as the type 1 and type 2 inhibitors. The type 1 includes simvastatin, pravastatin and lovastatin and is characterized by a decalin structure and a butyryl group apart from the HMG-like portion. Type 2 inhibitors, which are atorvastatin, cerivastatin and fluvastatin, are distinguished by a fluophenyl group instead of a butyryl group and a methylethyl group in place of the decalin ring, which leads to additional interactions [52]. These various statins with different structures have diverse pharmacokinetics properties.
Figure 2.6: HMG-CoA reductase inhibitor: Type 1 (Simvastatin), Type 2 (Fluvastatin).
2.4.3 Pharmacokinetic Properties of Statins

Pharmacokinetics is the study of the action of drugs involving processes like absorption, distribution, metabolism and elimination. Even though all the above statins have cholesterol lowering properties, they differ in terms of drug interactions, bioavailability and whether it is hydrophilic or lipophilic in nature as shown in Table 2.1.

Table 2.1: Different pharmacokinetics properties of statins [53-59]

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Atorva Statin</th>
<th>Ceriva Statin</th>
<th>Fluva Statin</th>
<th>Lova Statin</th>
<th>Prava Statin</th>
<th>Simva Statin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro drug absorption (%)</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>12</td>
<td>60</td>
<td>24</td>
<td>&lt;5</td>
<td>17</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Protein binding (%)</td>
<td>≥ 90</td>
<td>&gt;99</td>
<td>98</td>
<td>&gt;95</td>
<td>50</td>
<td>95</td>
</tr>
<tr>
<td>Excretion – Urine &amp; feces (%)</td>
<td>99</td>
<td>94</td>
<td>95</td>
<td>93</td>
<td>90</td>
<td>73</td>
</tr>
<tr>
<td>Half-life (hours)</td>
<td>14</td>
<td>2-3</td>
<td>&lt;1</td>
<td>3-4</td>
<td>1.8</td>
<td>3</td>
</tr>
<tr>
<td>Inhibition of VSMC</td>
<td>Direct</td>
<td>Immune</td>
<td>Immune</td>
<td>Immune</td>
<td>Immune</td>
<td>Direct</td>
</tr>
<tr>
<td>(With respect to potency)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP substrate</td>
<td>CYP3A4</td>
<td>CYP3A4CYP2C9</td>
<td>CYP3A4</td>
<td>Sulfation</td>
<td>CYP3A4</td>
<td></td>
</tr>
<tr>
<td>Lipophilicity</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

2.4.3.1 Absorption

After oral administration, the drug is taken into the bloodstream by absorption. The extent to which the drug is absorbed into the bloodstream is the bioavailability. Simvastatin and lovastatin are the only two statins administered in the prodrug lactone
form as seen from Table 2.1. They are inactive and are hydrolyzed either chemically or enzymatically to form respective active beta hydroxy acid forms [60,61].

The interconversion kinetics has not been yet characterized in either humans or animals. The enzyme hydrolysis takes place by esterase activity in the liver and plasma as well as in intestinal mucosa [62]. Lactone to acid conversion occurs by hydrolysis. It has been observed that, conversely, lactonization of statin acids takes place either by acyl glucuronide (glucuronidation) or by CoASH dependent pathway [63]. Both these processes are reversible though not in equal measures. The rate of hydrolysis of simvastatin (SV) to simvastatin acid (SVA) is 10 fold higher than SVA to SV conversion, which suggests that this process favors formation of SVA. First pass hepatic extraction has been noted to be higher for the lactone form of simvastatin than for simvastatin hydroxy acid (SVA) thereby reducing the systemic exposure of drug in the circulation [61].

CoASH is coenzyme A which might be attached to either an acetyl molecule (acetyl-CoA) or sulfahydryl group (-SH). Glucuronidation is a detoxification pathway wherein a sugar moiety is conjugated with the drug. This facilitates the reduced toxicity of drug along with elimination. The irreversible elimination clearance (IEC) of lactones to their metabolites is achieved by CYP450 mediated oxidation (Figure 2.7) wherein the IEC is the amount of drug irreversibly eliminated from circulation [63,64]. The acid clearance is attained by beta oxidation and CYP450 mediated oxidation.
2.4.3.2 Distribution

The transportation of the drug to the target sites depends on its ability to cross membranes after being absorbed. Lipophilic drugs include simvastatin, lovastatin and atorvastatin and are set apart from the hydrophilic ones like pravastatin. These lipophilic drugs can combine or dissolve in lipid based environments. Fluvastatin is unique by being in the intermediate between lipophilic and hydrophilic. It is twice as hydrophilic as lovastatin but 40 times more lipophilic than pravastatin. The open hydroxy acid forms of simvastatin, lovastatin, atorvastatin are 100 times more lipophilic than pravastatin. This
lipophilic nature has been suggested as the reason for the passive diffusion through extrahepatic cells and other cellular membranes that might account for higher inhibition of sterol synthesis in addition to inhibition of vascular cells. The more hydrophilic pravastatin has been observed to have an equal or higher HMG-CoA reductase activity in the liver. This might be due to a specific transporter, which enables uptake of pravastatin into the hepatocytes, the main structural component of the liver [65].

Apart from the ability to cross membranes, the capability of the drug’s binding to proteins plays a major role in transportation. As seen from Table 2.1, almost all statins with the exception of pravastatin (50%) are tightly bound (>90%) to proteins, especially albumin. This is one of the reasons as explained by Horsmans et al [66] that pravastatin is preferred for albumin bound drugs like warfarin, as there is less likely chance of displacement.

2.4.3.3 Metabolism

After distribution of the drug in the body, metabolism takes place. The chemical alteration of the drug in the body is termed as metabolism and the substances resulting from it as the metabolites. As mentioned earlier, prodrugs are converted to active metabolites which then have the desired therapeutic effects. Almost all statins are metabolized through the cytochrome CYP450 which is a family of 100 enzymes. There are two large categories- the steroidogenic and the xenobiotic enzymes. The former is not engaged in the metabolism of drugs while the latter is sub divided into four major enzyme families- CYP1, CYP2, CYP3, CYP4. Out of these, CYP3A4 is involved in the metabolism of atorvastatin, lovastatin, simvastatin. Cerivastatin is jointly metabolized by
CYP3A4 and CYP2C8 and fluvastatin is only by CYP2C9 (Table 1). Pravastatin is the only exclusion as it is metabolized by sulfation process.

2.4.3.3.1 Drug Interactions

Numerous drug interactions with statins are determined chiefly by CYP3A4 metabolism. This is because of the fact that CYP3A4 is involved in the metabolism of several drug medications. Higher affinity of a certain drug to the enzyme as compared to that of statin prevents the statin from binding to the enzyme [67]. This subsequently inhibits the metabolism. Accordingly, simultaneous binding of statin with another drug will consequently result in lower metabolism too. The drugs that fall under the category of CYP3A4 and CYP2C9 inducers are rifampin, phenobarbital, phenytonin and carbamazepine. Inhibitors of CYP3A4 isoenzyme include calcium channel blockers, erythromycin and cyclosporine and that of CYP2C9 inhibitors are ritonavir and omeprazole. Histamine-2- blockers and azole antifugals are inhibitors of both CYP3A4 and CYP2C9. Since most of statins are metabolized via CYP3A4, the drugs like fluvastatin and pravastatin run a less likely risk for drug interactions as they are metabolized through CYP2C9 and sulfation respectively. Fluvastatin was shown by Lanjung and Markhum [68] to have no considerable drug interaction effects with CYP2C9 drugs like histamine 2 blockers, omeprazole and cyclosporine.

Drug-food interactions do occur but they are clinically not significant except for grapefruit juice, which is an inhibitor of CYP3A4 isoenzyme. It has been shown to have significant effect on simvastatin, atorvastatin and lovastatin though not much on pravastatin [50].
2.4.3.4 Elimination

The final step is the process of elimination, wherein the drug is excreted from the body mostly in the form of feces or urine through the kidney. Water soluble drugs and drugs not tightly bound to proteins are expelled via urine and the others through feces. The kidney's ability to expel the drugs is dependent among other things, on the condition of the kidneys. If renal impairment occurs, the function of the kidneys decreases. This reduces the capability of drug excretion and there is an accumulation of metabolites and drug, upon which dosage adjustment needs to be made. Simvastatin and lovastatin dosage levels along with pravastatin and cerivastatin has shown a higher concentration compared to atorvastatin and fluvastatin in renal impaired patients [53]. The safety and tolerability levels of statins are dependent on the various pharmacokinetic properties especially that of drug interactions and drug levels in the liver.

2.4.4 Safety of Statins

Statin monotherapy has a few unfavorable effects as in hepatic toxicity and myopathy. There has been an increase in serum liver transaminases (3X higher than normal) where serum transaminase or alanine transaminase is an enzyme in the hepatocyte, which leaks into the plasma when the liver cell is damaged. Nonetheless, after discontinuation of the drug, the levels fall back to normal [69]. The other effect is myopathy, which can cause tenderness and weakness of the affected muscle, where there is an increase in creatine kinase level (10X higher than normal) [69]. Creatine kinase is an enzyme concentrated mostly in the muscle tissue and a rare condition of myopathy is termed rhabdomyolysis - the breakdown of muscle fibers leading to the release of muscle fiber contents into the circulation. This harmful effect caused by statin monotherapy is
very low but is usually elevated by taking other medications (combination therapy) and is also dose related. Cerivastatin has been the exception as it has been lately banned from the US market. This is because of its adverse effects with the respect to drug interactions and increased rhabdomyolysis [70]. These major adverse effects of statins, including hepatic toxicity and myopathy are addressed by use of the localized controlled drug delivery system.

Dose titration can prevail over reduced statin levels in case of the inducers of CYP3A4 and for the inhibitors of CYP3A4, toxicity occurs due to the elevated level. Another reason for dosage adjustment is the renal failure or impairment which then leads to dose reduction. Some of the medications, which affect the HMG CoA reductase inhibitor simvastatin, are erythromycin, cyclosporine, gemfibrozil, verapamil and grapefruit juice to name a few [67]. The other side reactions are constipation, nausea, fatigue and headache which were observed to be generally mild and transient. Overall, statins are well tolerated in clinical studies and have proved to have many other autonomous effects apart from lowering cholesterol levels.

2.4.5 Pleiotropic Effects of Statins

Statins have effects on the vascular wall independent of the ability to lower cholesterol. Inhibition of the mevalonate pathway has downstream consequences of reduced thrombogenicity, marked reduction of inflammation and improved vasodilation. The effects of statins on endothelial function, plaque stability, inflammation and on vascular smooth muscle cells are all interconnected (Figure 2.8) to have a marked change in coronary heart disease, arteriosclerosis and other vascular developments.
2.4.5.1 Endothelial Function

The endothelium is a thin layer of flat cells situated in the intima, lining the surface of the blood vessels. The endothelial functions are inhibition of platelet activity, thromboresistance, fibrinolysis and regulation of the vasomotor tone. For all these functions, the endothelia synthesizes nitric oxide, endothelin-1 (ET-1), prostaglandins and tissue-type plasminogen activator (t-PA) and cytokines. Endothelial dysfunction might occur due to restenosis leading to reduced inhibition of all the above factors. Statins improve the endothelial function by increasing nitric oxide (NO), which is done by upregulation of endothelial NO synthase (eNOS). This was shown in the study conducted by Liao, Fata and Laufs [71] wherein simvastatin and to a lesser extent lovastatin prevented the down regulation of eNOS caused due to hypoxia. In a separate study by Perera et al [72], atorvastatin and simvastatin augmented ET-1 and eNOS which led to the inhibition of smooth muscle cell in addition to vasodilation and decreased thrombotic activity.
Figure 2.8: Pleiotropic effects of statins. eNOS- endothelial nitric oxide synthase, NO- Nitric oxide, t-PA- tissue type-plasminogen activator, TXA$_2$ – thromboxane $A_2$
2.4.5.2 Platelet Activity and Plaque Stability

As briefly explained earlier, platelet activation leads to PDGF, as well as the production of thromboxanes especially thromboxane A₂ synthesis, which plays a major role in platelet aggregation as it paves the way for thrombosis. Simvastatin, along with pravastatin was found to inhibit platelet function and thromboxane A₂ considerably, though the exact mechanism is unknown [73]. The secretion of PDGF in turn produces the proliferation of macrophages and smooth muscle cells. Macrophages produce foam cells, matrix metalloproteinases (MMP) and the cytokines. The production of MMP leads to the rupture of plaque cap while that of cytokines to T-lymphocyte proliferation. Now plaque stability is an important factor to be considered as the rupture of it causes acute coronary syndrome (ACS) [74]. ACS is defined by American Heart Association (AHA) as a term used to cover any group of clinical symptoms compatible with acute myocardial ischemia and includes unstable angina, non-Q-wave myocardial infarction and Q-wave myocardial infarction. Statins have proved to reduce the macrophage growth [75] thus steadying the plaque.

2.4.5.3 Vascular Inflammation

Interventional procedures lead to inflammation factors like secretion of interleukins (from cytokines) and T lymphocytes which basically modify endothelial function, platelet and smooth muscle cell growth. Intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) participate in the leukocyte-endothelium interaction [76]. High sensitivity C-reactive protein (CRP) is a marker of inflammation produced in response to pro-inflammatory cytokines like IL-6 and is a low grade systemic
inflammation. Lovastatin [77], pravastatin [78] have shown effects on natural killer cell functions.

In a study by Niwa et al [79], fluvastatin reduces the adhesion molecule expression and in so doing lowers the vascular inflammation. Simvastatin was demonstrated to have little effect on VCAM-1, ICAM-1 alone but CRP protein levels were significantly lowered [80] by both simvastatin and atorvastatin [81].

2.4.5.4 Smooth Muscle Cell Proliferation

Intimal hyperplasia is characterized by smooth muscle cell growth. The pathway for VSMC has been discussed in detail earlier in the section 2.4.1. Briefly, in the mevalonate pathway, isoprenoid intermediates like GGPP and FPP are lipid attachments for the posttranslational modification of proteins like Ras and Rho GTP binding proteins. The Rho family is involved in the reduced eNOS activity in addition to the smooth muscle cell production. Statins have proved that inhibition of the isoprenoid products in the MVA pathway has lead to the down regulation of p27kip1, Cdk inhibitor involved in the phosphorylation of Rb[41]. This subsequently results in the prevention of migration and proliferation of smooth muscle cells. eNOS also was found to increase as shown by Laufs U et al [82].

It has been proved by two independent studies that lovastatin, simvastatin, atorvastatin and fluvastatin have dose dependently lowered smooth muscle cell migration but inhibition by pravastatin was very low [58,83]. This might be due to the fact that pravastatin is a hydrophilic drug and has less diffusion through extrahepatic cells. In a recent study by Breummer et al [55] it was discussed that simvastatin more potently inhibited global phosphorylation of Rb as compared to atorvastatin. The reason for more
global effect was that atorvastatin only disrupted the binding of Rb. On the contrary, simvastatin apart from Rb disruption, promoted the release of E2F. This is a transcription factor for the DNA synthesis which leads from S phase to M phase.

To summarize, statins including cerivastatin, lovastatin, fluvastatin and pravastatin all primarily inhibit the smooth muscle cell proliferation through the inflammatory cytokines as well as T-lymphocytes. Atorvastatin and simvastatin have a more direct approach due to the global phosphorylation effect. Also in a study done by Corsini et al [84], the IC$_{50}$ values, which are the concentration of the drug needed to inhibit cellular proliferation by 50% were found to be greater for atorvastatin than simvastatin. The cholesterol independent effects of simvastatin show it to have an overall inhibitory effect for the coronary system thereby making simvastatin the choice of drug for the study.

2.5 Current State of the Art Device- Vascular Wrap

A brief mention has to be made about a new state of the art drug delivery device, Vascular Wrap™. This device manufactured by Angiotech, a Canadian based pharmaceutical company was recently undergoing clinical trials for the prevention of intimal hyperplasia. This device closely resembled the PolyRing device and is thus of particular significance.

The Vascular Wrap is a paclitaxel eluting mesh for preventing stenosis after peripheral bypass graft surgery. Paclitaxel, a hydrophobic drug, has been proved to be quite efficient in prevention of intimal hyperplasia [85,86] and in cancer therapy [87,88]. The bioresorbable, biodegradable device consisted of a PLGA polymer coated with PEG polyester containing paclitaxel which was wrapped around the anastomosis site after graft
surgery was performed. In the results observed, the wrap was found to be very effective in reducing intimal hyperplasia (1.6 mg, -84% stenosis) along with reduced death rates due to adverse events (11%) as compared to controls (18%) [89,90].

Limited knowledge and data available for the Vascular Wrap made the assessment of the two different device systems as a matter of interest, challenging. The drug of choice and the means by which intimal hyperplasia was inhibited was found to be of significance. Though both simvastatin and paclitaxel could prevent VSMC proliferation, their mechanism of action was quite different. In short, simvastatin acted as a HMG coA reductase inhibitor in the mevalonate pathway preventing the formation of prenylated proteins which were involved in cell proliferation and migration. In depth study of the mechanism has been mentioned elsewhere in the document. Paclitaxel mechanism of action was through the microtubules. Microtubules form structural components within cells and were responsible for cellular processes including mitosis and vesicular transport. The microtubules consisted of α and β tubulin dimers which underwent polymerization and depolymerization leading to dynamic instability. The instability was the shortening and lengthening of microtubules which was necessary for the transportation processes. Paclitaxel by means of binding to the β subunit stabilized the microtubules resulting in loss of depolymerization, loss of shrinkage and possible cell death [87]. A fact to be regarded for the action of simvastatin was, it was reversible that is if stopped, would not affect normal VSMC proliferation and migration. Whereas with paclitaxel if stopped, as cell death had already occurred, the process could be irreversible and may result in higher cell death count than required.
The structures of the two devices, along with the treatment strategy were quite significant. While in the PolyRing, the drug was encapsulated in the microspheres and then embedded in the block polymer (Figure 2.2), in the Vascular Wrap the paclitaxel was in a mesh sheet like structure (Figure 2.9). This could lead to a much more controlled and slower release in the former. As for the latter, it could have a higher release rate including a higher burst effect on account of the higher surface area leading to faster depletion of the drug. Surface area of the device could be a vital parameter for therapeutic efficiency. There could be drug loss due to the absorption of drug into the extra arterial capillaries, a pathway for drug transportation.

Figure 2.9: Schematic illustration of Vascular Wrap [91]
The treatment strategy for the PolyRing was that the ring could be clamped snugly directly around the areas surrounding the graft surgery conforming to the shape of the artery with a capability of multiple rings placements (Figure 2.10). The Vascular Wrap was placed roughly over the surgery site leading to a greater possibility of tearage and breakage. Though the larger wrap depending on the distribution of the drug could theoretically deliver over a higher section of the anastomosis site, the breakage mentioned above could displace the device rendering it inefficient.

The types of graft along with the drug play an essential role in effectiveness of the device. The Vascular Wrap has been currently marketed to be used exclusively with Lifespan ePTFE Vascular Graft while the PolyRing has no such restrictions and was devised with no particular graft in mind.
CHAPTER III
MATERIALS AND METHODS

3.1 Materials

Poly (ethylene glycol) (PEG) of 3350 molecular weight was bought from Sigma (Sigma-Aldrich Co, St Louis, MO, USA). Dichloromethane (DCM) was purchased from Aldrich (Sigma-Aldrich Co, Milwaukee, WI, USA). Simvastatin ZOCOR ® was a gift from Merck (Merck & Co., Inc, West Point, PA, USA) and poly (DL lactide co glycolide) 85/15 (PLGA) was obtained from API (Absorbable Polymers International, Birmingham, AL, USA). Poly (vinyl alcohol) (PVA) was purchased from Sigma Aldrich. Triphenyl methane triisocyanate (Desmodur RE) was received as a gift from Bayer (Bayer Corporation, Pittsburgh, PA, USA). All materials obtained were used as received without purification.

3.2 Device Fabrication

The PolyRing device fabrication followed a step by step procedure in which first the PLGA microspheres were prepared by oil in water (o/w) emulsion technique in which the simvastatin was encapsulated. After which the drug loaded PLGA microspheres were embedded in the PEG hydrogel block polymer and drilled to get the desired PolyRing device. This was followed by sterilization by hydrogen peroxide.
3.2.1 Preparation of PLGA Microspheres

Microspheres were prepared by the modified solvent evaporation o/w emulsion technique as described by Kanjickal et al [9,10]. Briefly, the organic phase constitutes 1g of PLGA dissolved in 15 ml of dichloromethane (DCM) along with 0.1 g of simvastatin. The organic phase was added slowly to the aqueous phase which has 0.5% PVA solution and continuously stirred with the help of a stainless steel impeller (LR400D, Yamato Scientific Co Ltd, Tokyo, Japan) for 60 minutes at a constant speed of 350 rpm and room temperature. The resulting solution was then processed through a rota-evaporator for the evaporation of the solvent and then filtered using distilled water and finally freeze-dried (Labconco Corporation, Kansas City, MO, USA) for three days. This then yielded the PLGA microspheres loaded with the simvastatin drug. Blank PLGA microspheres were prepared in a similar fashion except for the lack of drug solution in the organic phase.

3.2.2 Preparation of the PolyRing Device

The poly (ethylene glycol) PEG hydrogel was prepared by chemical crosslinking of PEG with triphenyl methane triisocyanate (Desmodur RE), a cross linking agent. In brief, 25% (weight) dichloromethane solution of Desmodur RE and 25% (weight) solution of PEG, molecular weight 3350 were prepared. 21 g of Desmodur RE solution was added to 75 g of PEG solution for the desired cross linking ratio of 1:1. The resulting mixture then was stirred continuously at a temperature of 70°C for half an hour to get a fairly viscous solution. After which, 1 g of PLGA microspheres suspended in 18 g of DCM was poured into the PEG mix and the whole mixture was transferred into aluminum pans (size of 5.8cm, depth of 0.7cm) in order to obtain the hydrogel block polymer [9,10]. The resulting polymer was allowed to stand for a period of 24 h at 70°C. After which the
polymeric rings of approximately 5mm OD and 3mm ID were extracted from the block polymer with the help of a drill.

3.2.3 Device Sterilization

Sterilization of the PolyRing device was done by hydrogen peroxide in a Sterrad 100S sterilizer (Johnson and Johnson). Sterility is defined as the state of being free of pathogenic organisms. This property is very important for biomaterials, which might come into contact with the body. The Sterrad equipment uses the hydrogen peroxide gas plasma process technique to obtain $10^{-6}$ Sterility Assurance Level (SAL). SAL is the expected probability of an item being non sterile after a sterilization procedure. A SAL of $10^{-6}$ means that there is a probability of one in a million that the item is nonsterile. Hydrogen peroxide is an oxidizing agent, which also has bactericidal, virucidal, sporicidal and fungicidal properties at low concentrations and temperature. The gas plasma is highly ionized gases made up of ions, electrons and neutral particles. The sterilization process works as follows:

The liquid chemical agent hydrogen peroxide and water with 59% nominal $\text{H}_2\text{O}_2$ by weight is vaporized and interaction takes place with the device to be sterilized in a circular chamber. When a strong electrical field is applied, plasma is formed which then subsequently breaks down the peroxide and forms a cloud of highly energized matter. This recombines with the peroxide and by products obtained are water vapor and oxygen. The total cycle time is approximately 55 minutes and the temperature is $< 55 ^\circ \text{C}$. 

42
3.3 Characterization of the Microspheres

The blank and drug loaded microspheres were characterized for surface morphologies, particle size distribution, drug loading and encapsulation efficiency. The methodology for determining all of the above are described in this section.

3.3.1 Microsphere Surface Morphology

The surface morphology of the blank microspheres and the drug loaded microspheres was observed with the aid of the scanning electron microscope (SEM) (Joel JSM-35C, Jeol Ltd, Tokyo, Japan). The samples were prepared as follows: double stick black conducting tape was applied on a clean sample stub and a thin coating of the sample microspheres was spread on it. After which, the sample was sputter coated with silver at 25kV so as to provide a conductive layer. The sputter coated sample was then loaded into the chamber of the microscope and then voltage increased slowly from 0.5 kV to 20kV. The magnification was adjusted till a clear image of the surface of the microspheres appeared and the picture was then recorded.

3.3.2 Particle Size Analysis

Microsphere sizes were determined using two different techniques: the optical microscope analysis for achieving the 100µm diameter microsphere size by varying the impeller speed and the light scattering method for determining the microsphere sizes for the different runs.

3.3.2.1 Optical Microscope Analysis

In order to obtain the desired microsphere diameter of 100µm, the impeller speeds varied were 450 rpm, 350 rpm, 300 rpm and 250 rpm. The different samples were
evaluated as follows: A fine layer of the microspheres was placed on the specimen stage which was raised to be positioned under the microscope objective. The desired magnification was achieved and the images recorded. The size distribution range was measured by manual counting of the microspheres. Briefly, the method of counting was done by first taking several photographs (seven) of the microspheres. The diameter was then established by measuring each microsphere in a photograph with a scale of approximately 4.4cm equal to 100 microns determined by means of measurement scale. This process was repeated for each process speed and the speed which yielded a narrow size distribution for 100µm microspheres was chosen to be the fixed impeller speed for the remainder of the experiments.

3.3.2.2 Light Scattering Analysis

Particle size analysis was determined by a static light scattering (SLS) method based on Fraunhofer and Mie theories (Coulter Particle Size Analyzer LS 100Q, Beckman Coulter, Inc, Fullerton, CA, USA) with a suspension of microspheres. The principle behind SLS method was that the largest to the smallest particle is taken into account. When the particle size is bigger than the wavelength of incident light, light scattering patterns in the forward direction are caused by small diffraction angles- Fraunhofer diffraction. For a particle size smaller than the wavelength of light, the patterns are scattered sideways and back by refraction and absorption- Mie scattering.

Sample preparation was done as follows; 10-50 mg of the freeze dried microspheres was added to a vial containing a freeze-dried diluent cake. The diluent was composed of 91% mannitol, 7.5% carboxymethyl cellulose (CMC) and 1.5% Tween 80 by weight.
The mannitol serves as a lyo-protectant, the CMC as a viscosity modifier to prevent settling of the microspheres and the Tween 80 acts as a surfactant. Degassed, deionized water (1 ml) was added to the vial and swirled to reconstitute the diluent and microspheres. This was then sonicated for 2 minutes to break-up any aggregation.

The fluid module of the particle sizer (where sample loading takes place) was washed with degassed, deionized water to get rid of the previous particles and bubbles. Degassed water was obtained by passing deionized water through a 0.45 μm filter under vacuum. Following the washing step, the water was filled in the fluid module and the mixing pump was switched on at 35% speed. A control sample (a well-defined particle size) was run at the beginning of each day. The control standard’s median value should be within ± 5% of the value listed on the Standard’s Certificate of Analysis. After the washing and refilling of the fluid module, the suspension of microspheres was loaded gradually with a transfer pipette so as to not go above the window of obscuration. The obscuration should be in the range of 8-12% for diffraction samples. The results are determined by the average of 3 analyses per sample. In between sample measurements, the equipment is carefully rinsed with water three times. The mean diameter and the standard deviation were recorded.

3.4 Drug Loading Studies

The drug loading studies including the actual loading and the encapsulation efficiency of the simvastatin loaded microspheres were determined. The amount of statin encapsulated in the microspheres was measured by Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) system described below. A known amount of freeze-dried
drug loaded microspheres (3mg) was dissolved in 30 ml of acetonitrile and was shaken gently for 30 minutes. After which the sample was filtered through a 0.45mm syringe filter (Becton Dickinson & Company, Franklin Lakes, NJ, USA) to filter out any wanted particulates and then injected into the HPLC system. Encapsulation efficiency was determined by the following formula:

Encapsulation Efficiency (EE) percentage

\[
\text{Encapsulation Efficiency (EE) percentage} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100
\]

The drug concentrations for the actual drug loading were established by comparing the peak areas to a standard calibration curve which was verified by a series of known concentrations of drug in acetonitrile.

3.4.1 Reverse Phase High Pressure Liquid Chromatography

The HPLC was used to determine the actual loading of the drug. The HPLC (LC-10AT Shimadzu Corporation, Kyoto, Japan) arrangement consists of a continuous phase and a mobile phase. The mobile phase acts as a carrier for the sample solution. The columns are packed with the stationary phase, which was immobile and immiscible to the mobile phase forced through it. There are several columns containing stationary phases. One of them was the Reverse Phase column in which retention is the result of the interactions between non polar components of the solutes and the non polar stationary phase. Retention time was defined as the time elapsed from the introduction of the sample to when there is a maximum response.
The HPLC system utilized consisted of a pump, an auto sampler and a UV detector. The stationary phase had silica based packings and the tendency of the column was to retain the hydrophobic compounds. Therefore the more hydrophilic moieties elute faster than the hydrophobic ones. The detector was the part located posterior to the stationary phase, which sends out a response of the eluting compound in the form of a peak. The dispersion reverse phase silica column (Aquasil C18) provided by Thermo Scientific (Thermo Scientific Inc, Waltham, MA, USA) was used for the separation process. The separation was done at room temperature at the flow rate and the UV wavelength of 1.2 ml/min and 237nm respectively. The mobile phase employed was an acetonitrile, water (70:30) system.

3.5 In Vitro Release Studies

The release studies of the PolyRing, which has the PEG block polymer dispersed with the drug loaded microspheres, were performed in amber glass bottles containing 10 ml phosphate buffered saline (PBS) pH 7.4 as the release medium. The samples were positioned in a shaker having a constant agitation at 60 rpm and kept in an incubator at 37 °C. At regular intervals of 1h, 4h, 8h, 24h, 3rd day, 5th, 10th, 14th, 21st and 28th days, 1 ml of sample was removed and fresh buffer replaced to the amount removed. The samples were then filtered through the same 0.45 mm syringe filter and HPLC analysis conducted. Release studies were performed for unsterilized and hydrogen peroxide sterilized samples.
CHAPTER IV
RESULTS AND DISCUSSION

The purpose of the present research was to modify an already established system using the drug, simvastatin to inhibit IH. The microspheres were synthesized by the oil in water (O/W) emulsion procedure. In this method, the drug was dissolved into an organic solution, which was then emulsified into an aqueous external phase, forming droplets. After solvent removal and polymer precipitation, the microspheres were formed. The purpose after the synthesis of the simvastatin loaded microspheres was to characterize the surface morphology, the particle size distribution and drug loading. In particular, the effect of various parameters including drug to polymer ratio, emulsification time, surfactant concentration and aqueous volume on drug loading and encapsulation efficiency was ascertained. Finally, simvastatin loaded microspheres were suspended in the PEG hydrogel block polymer which was drilled to form the PolyRing device.

4.1 Microsphere Characterization

Figures 4.1 and 4.2 illustrate the morphological characteristics of both the blank microspheres and the simvastatin-loaded microspheres obtained by SEM. The microspheres were spherical with smooth surfaces and no drug crystals observed on the surface of any of the various formulations. The loading of the drug did not lead to any
significant change in the morphological characteristics of the correspondingly empty microspheres. Figure 4.3 shows a polyanhydride microsphere with drug crystals found on the surface [92], to illustrate this undesired distribution.

Figure 4.1: Scanning electron micrograph of blank PLGA microspheres
Figure 4.2: Scanning electron micrograph of simvastatin loaded PLGA microspheres

Figure 4.3: Scanning electron micrograph of rhodamine loaded polyanhydride microspheres [92].
4.2. Optical Microscope Analysis of Blank PLGA Microspheres

The objective of determining the impeller speed was to obtain a microsphere diameter of 100 µm which was the optimized size established for the previous system. The impeller speed, which is the operating parameter, was varied with different speeds including 450 rpm, 350 rpm, 300 rpm and 250 rpm. With four different speeds, there was a diameter range from 120µm to 80µm obtained from manual counting (Figure 4.4).

![Graph showing the effect of various impeller speeds on blank microspheres diameter by optical microscopy. Data are moving average of three.](image)

Figure 4.4: Effect of various impeller speeds on blank microspheres diameter by optical microscopy. Data are moving average of three. ◆ 450 rpm, △ 350 rpm, ■ 300 rpm, ▲ 250 rpm.

Increased diameter size (120µm) was obtained for 250 rpm and as the speed increased to 450 rpm the microsphere size decreased (80µm). This was because as the impeller speed increases, it imparts more shear stress to the emulsion thus forming smaller droplets. The graph shows the data as the moving average of three along with the
trendline. Thus 350 rpm speed was found to be the most suited for the required diameter of 100 µm.

4.3 Drug Loading Analysis

One of the aims of this study was to examine the significance of the preparation parameters of the o/w emulsion method with respect to drug loading and encapsulation efficiency. Further, it was to establish the maximum encapsulation efficiency of the drug loaded microspheres. The design used to get the best formulation parameters of drug loaded microspheres, by using different formulation conditions, was a $2^{4-1}$ fractional factorial design (FFD). The standard parameters considered while preparing microspheres in the o/w emulsion method include the drug to polymer ratio (A), the surfactant concentration (B), the emulsification time (C) and finally the aqueous volume (D). Each of these four factors has two levels, a High (H) and a Low (L) which represent the qualitative range of that particular factor wherein there is an appreciable change in the result. Normally, the full factorial design will be $2^4$ which is equal to 16 runs. In this case, running one half fraction of the complete factorial design (8 runs where $D=ABC$) is enough to provide information on the main effects and the lower order interactions. If necessary the other one half fraction (remaining set of 8 runs where $D=-ABC$) can be completed to get the complete design [93]. FFD is fundamentally used for screening purposes, the rationale being that with several variables, one would be able to identify factors that have significant effects. Basic assumptions when using a FFD are that all the variables are fixed, completely randomized and all higher order interactions are considered negligible.
Table 4.1: Experimental design of a $2^{4-1}$ fractional factorial design

<table>
<thead>
<tr>
<th>RUNS</th>
<th>Factors</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Drug to Polymer ratio (g of drug/g of polymer)</td>
<td>Surfactant concentration (%)</td>
<td>Emulsification time (min)</td>
<td>Aqueous Volume (ml)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>0.5</td>
<td>30</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>0.5</td>
<td>30</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>1</td>
<td>30</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>0.5</td>
<td>60</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>0.5</td>
<td>60</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>1</td>
<td>30</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>1</td>
<td>60</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.2</td>
<td>1</td>
<td>60</td>
<td>170</td>
<td></td>
</tr>
</tbody>
</table>

KEY

- **Low**
- **High**

Eight experiments have been carried out according to the fractional factorial design $2^{4-1}$ with two levels, four factors as demonstrated in Table 4.1. The experiments were conducted in fully randomized order. The encapsulation efficiency (EE) is defined as the percentage of actual drug loading to theoretical drug loading. Drug loading is amount of drug loaded into the microspheres. The drug encapsulation experiments were performed in triplicate for each run and thus the data obtained was the average of three measurements. A particle size analysis was run after the set of experiments to determine the microsphere size.
4.3.1 Particle Size Analysis using Light Scattering Method

Figure 4.5 shows the particle size distribution of the statin loaded microspheres, which was obtained by static light scattering. The results obtained were the average of 3 analyses per run. This method was done to determine the size of the microspheres and to check whether there was any significant change in the diameters of the different runs in the experimental design. The size of the particle was an important characteristic of the microspheres as it could affect drug loading properties during article preparations especially with respect to encapsulation efficiency. In general, as size increases, there was a slower precipitation or hardening which decreased the encapsulation efficiency. This was again due to the partitioning of the drug to the aqueous phase. The particle size analysis for the microspheres diameters was in the range of 94 μm to 120μm (Figure 4.6). There were no statistical differences in the diameter of the various runs. This lead to the observation that size of the drug loaded microspheres was not affected by the four parameters taken into consideration while preparing the same. A Tukey test was used, for comparing the diameters of the different runs, at significant levels of 95 %. This post priori test was particularly used to compare the various diameters of the runs and also as it is most conservative of such tests including Student Neuman Keul (SNK) test and Duncan test.
Figure 4.5: Graph of particle size distribution of Run 4 as optimized from the experimental design table 4.2.

Figure 4.6: Particle size distribution of eight runs as observed in the experimental design Table 4.2.
4.3.2 Actual Drug Loading and Encapsulation Efficiency

The actual drug loading, the mean encapsulation efficiency displayed as mean EE ± S.D and the mean diameter are shown in Table 4.2. The influence of each variable was evaluated by the analysis of variance (ANOVA) and the effects of each of the four factors on encapsulation efficiency were found out to be either significant or not. Seen from Table 4.3, the two factor interactions are seen to have their own aliases, which come to a total of six, two factor interactions. This follows the resolution IV design where the main effects like A, B, C and D are not aliased with other main effects or two factor interactions but two factor effects are aliased with each other. Two or more effects which have the same properties are termed aliases. For example, with respect to two factor interactions, it is impossible to differentiate between AB, CD; AC, BD and BC, AD. This is represented by AB+CD, AC+BD, BC+AD [93].
Table 4.2: Encapsulation efficiency, drug loading and mean diameter

<table>
<thead>
<tr>
<th>RUNS</th>
<th>Actual Drug Loading (μg drug/mg of microsphere) ± S.D</th>
<th>EE% ± S.D</th>
<th>Mean Diameter μm ± S.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75.11 ± 12.50</td>
<td>75 ± 13</td>
<td>92 ± 22</td>
</tr>
<tr>
<td>2</td>
<td>68.85 ± 3.80</td>
<td>34 ± 4</td>
<td>105 ± 23</td>
</tr>
<tr>
<td>3</td>
<td>74.60 ± 4.50</td>
<td>75 ± 5</td>
<td>110 ± 37</td>
</tr>
<tr>
<td>4</td>
<td>80.65 ± 8.80</td>
<td>81 ± 9</td>
<td>94 ± 23</td>
</tr>
<tr>
<td>5</td>
<td>68.80 ± 10.10</td>
<td>34 ± 10</td>
<td>108 ± 29</td>
</tr>
<tr>
<td>6</td>
<td>78.35 ± 2.80</td>
<td>39 ± 3</td>
<td>122 ± 65</td>
</tr>
<tr>
<td>7</td>
<td>78.26 ± 6.10</td>
<td>78 ± 6</td>
<td>101 ± 27</td>
</tr>
<tr>
<td>8</td>
<td>81.49 ± 6.30</td>
<td>41 ± 6</td>
<td>95 ± 26</td>
</tr>
</tbody>
</table>

Table 4.3: Analysis of variance. A- Drug to polymer ratio, B- surfactant concentration, C- emulsification time, D- aqueous volume

**ANOVA Table**

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>DoF</th>
<th>Mean square</th>
<th>F ratio</th>
<th>Fvalue (4.49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.9584</td>
<td>1</td>
<td>0.9584</td>
<td>230.0619</td>
<td>Significant</td>
</tr>
<tr>
<td>B</td>
<td>0.0025</td>
<td>1</td>
<td>0.0025</td>
<td>0.6029</td>
<td>N.S</td>
</tr>
<tr>
<td>C</td>
<td>0.0043</td>
<td>1</td>
<td>0.0043</td>
<td>1.0419</td>
<td>N.S</td>
</tr>
<tr>
<td>D</td>
<td>0.0004</td>
<td>1</td>
<td>0.0004</td>
<td>0.1071</td>
<td>N.S</td>
</tr>
<tr>
<td>AB+CD</td>
<td>0.0074</td>
<td>1</td>
<td>0.0074</td>
<td>1.7656</td>
<td>N.S</td>
</tr>
<tr>
<td>AC+BD</td>
<td>0.0022</td>
<td>1</td>
<td>0.0022</td>
<td>0.5292</td>
<td>N.S</td>
</tr>
<tr>
<td>BC+AD</td>
<td>2.7E-06</td>
<td>1</td>
<td>2.7E-06</td>
<td>0.0006</td>
<td>N.S</td>
</tr>
<tr>
<td>Error</td>
<td>0.0667</td>
<td>16</td>
<td>0.0042</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.0419</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
From Table 4.3 (ANOVA), it can be seen that only the drug to polymer ratio had a statistically significant effect (P≤0.05) on the encapsulation efficiency of simvastatin in the PLGA microspheres. The drug to polymer ratios examined were 10% and 20%. For better observation of the drug to polymer ratio effect on both drug loading and encapsulation efficiency, two sets of runs were differentiated, SET 1 comprising of 10% drug loading and SET 2 comprising of 20% loading.

Drug loading is the amount of simvastatin drug per milligram of PLGA microspheres. On examination, there was found to be no statistically significant differences between SET 1 and SET 2 in drug loading (Figure 4.7) with respect to drug to polymer ratio. It was observed that the actual drug loading for the microspheres ranged from 69 μg to 81 μg for the eight runs and the distribution was consistent within this range as seen in Figure 4.8. Therefore, the drug loading studies signified that only a certain amount of drug could be loaded into the microspheres beyond which any excessive drug remained in the solution.
Figure 4.7: Graph of the drug loaded microspheres of eight runs. SET 1 comprising of 10% drug loading (Runs 1, 3, 4, 7), SET 2 of 20% drug loading (Runs 2, 5, 6, 8)

Figure 4.8: Drug loading distribution of eight runs as observed in the Table 4.2
The encapsulation efficiency ranged from 34% to 81% for the eight runs. It was observed (Figure 4.9) that the 0.1 drug to polymer ratio (SET 1) had a significantly higher EE of the order of 75%-81% than the 0.2 drug to polymer ratio (SET 2), which had an EE of 34%-41%. When there was a higher drug content per milligram of microspheres, the encapsulation efficiency decreased. For both 0.1 and 0.2 drug to polymer ratio, since almost same amount of drug was being encapsulated in the microspheres, it resulted in more simvastatin diffusing from the organic phase into the aqueous phase. Thus more drug was present in the solution leading to drug loss. For the purpose of the study, which was to obtain the highest drug encapsulation efficiency, since only drug to polymer ratio had a significant effect, any run in SET 1 was considered feasible. Therefore, out of the set of formulation parameters, Run 4 which had the encapsulation efficiency of 81% with a 0.1 drug to polymer ratio, having a surfactant concentration (PVA) of 0.5% in an aqueous volume of 170 ml and a stirring time of 60 min at a constant stirring speed 350 rpm of the impeller was chosen for further studies.
4.4 In Vitro Drug Release Studies

The simvastatin loaded microspheres which had the highest encapsulation efficiency (81%) as seen in the above section, were suspended in dichloromethane and added to the PEG hydrogel to successfully yield the block polymer. This was further drilled to get PolyRings which were measured to be 5mm OD and 3 mm ID (approximately). Release studies were performed on unsterilized and hydrogen peroxide (H₂O₂) sterilized PolyRings to determine whether sterilization affects the release of simvastatin from the PolyRing device.

In general, the release profile of the controlled drug delivery system could be characterized by an initial rapid release of the drug termed as the burst effect. The burst effect takes place within a short period of time and leads to a higher release of drug
initially. This in turn could reduce the amount of drug retained in the device for a more controlled sustained delivery thus diminishing the effective lifetime of the drug along with the need of higher dosing. Some of the potential reasons for this effect could be attributed to surface characteristics of the material including the morphology, drug/polymer interactions and processing conditions [94]. Depending on the amount of drug available after the burst effect, the next phase was generally characterized by slow release of the drug resulting from degradation/erosion of the microspheres and diffusion of the drug. Final phase could be the complete erosion of the polymeric matrix and the subsequent increase in the drug release [95]

Simvastatin release from the unsterilized PolyRing device was conducted in 10 ml phosphate buffered saline (PBS), pH 7.4 shaken at 37°C in amber glass bottles. The ring achieved a maximum swelling of 8mm OD and 4mm ID (Figure 4.10) after four hours in PBS and the sampling times were inclusive of the swelling.
The RP-HPLC analysis for the simvastatin loaded microspheres dissolved in acetonitrile (mobile phase of acetonitrile and water (70:30)) yielded the retention times of simvastatin at approximately the 9th minute (Figure 4.11). On the other hand, the RP-HPLC chromatograms presented in Figure 4.12 show a peak at the 6th minute. This peak kept increasing over time. It was theorized to be simvastatin acid, the active metabolite of simvastatin. This is the open hydroxy acid form of the closed lactone form of simvastatin. The lactone drug may have been hydrolyzed and consequently simvastatin acid, which was soluble in the aqueous phase, may have been measured. The lack of a peak at the 9th minute might be due to the very low detection limits for simvastatin due to the low solubility of simvastatin in PBS (1.1µg/ml). [96, 97] This compares to a 20-fold higher solubility of the other hydrophobic drug studied by the group, cyclosporine A (27.67 µg/ml) [98]. This factor was not taken into consideration when the experiments were
started as CyA’s solubility was not called into question for the previous study. Only during the release studies was the solubility queried. After which, a series of experiments was done to ascertain whether the increasing peak might be that of simvastatin acid and to check if simvastatin can be detected even with low solubility.

Figure 4.11: Chromatograph for simvastatin loaded microspheres of Run 4 for the encapsulation efficiency study
To ensure that the peak noted at 6 minutes was not that of the PEG polymer, a blank PEG hydrogel block polymer, i.e. with no microspheres, was prepared and placed in the release medium. The results show the absence of a peak thus proving that it was not PEG being released. Furthermore, in an attempt to detect the simvastatin, the amount of PBS was reduced to 8ml and 6 ml from the original amount of 10 ml and the release measured. Though the peak at 6 minutes kept increasing, there was no peak detected for simvastatin drug at 9 minutes with reducing PBS. Another method employed for potential detection of simvastatin, was the mixing of 2ml of acetonitrile with 0.5ml drug release supernatant. This mixture was then injected into the HPLC system. The addition of acetonitrile was to help solubilize simvastatin if present in the sample. Also in 2 ml of PBS, 3 mg of drug loaded microspheres was added and was run through the chromatograph to check for particulates if any, from polymer degradation. Both these results confirmed the absence of simvastatin peak.
Therefore, these experiments which were done suggest that it might be simvastatin acid concentration increasing, and that the concentration of simvastatin was too low for detection. Hence, other new means have to be devised for the near future. These include the use of surfactants and protein mediated transport which will be explained in detail in the future work section.
CHAPTER V
CONCLUSION

Intimal hyperplasia is the leading cause of failure in interventional procedures. It is a wound healing response to these procedures which is characterized by the proliferation of vascular smooth muscle cells, which leads to the constriction of blood vessels thus reducing the flow of blood. Ultimately, this proliferation results in another set of surgeries, which is undesirable. A novel targeted perivascular polymeric based drug delivery system was developed by Kanjickal et al using cyclosporin A. Our research involved modifying the established system by using simvastatin, a HMG-CoA reductase inhibitor. Previous studies have found simvastatin to directly inhibit the proliferation of smooth muscle cells apart from being a cholesterol lowering drug. It inhibits the proliferation of smooth muscle cells by restraining the action of enzyme HMG-CoA reductase to prevent formation of mevalonate, a precursor to cell propagation and growth.

Simvastatin was successfully incorporated in the PLGA microspheres by O/W emulsion technique with a maximum drug loading of 81µg drug/mg of microspheres with no discernable drug crystals on the surface of the microspheres. Optimization of the processing parameters (drug to polymer ratio, surfactant concentration, emulsification time and aqueous volume) led to the highest encapsulation efficiency of 81%. The drug to polymer ratio had a statistically significant effect on the encapsulation efficiency
(p≤0.05). Thus the null hypothesis for the drug to polymer ratio having no significant
effect was rejected while the other three parameters; surfactant concentration,
emulsification time and aqueous volume; failed to be rejected as there was no significant
effects of these parameters on the drug encapsulation efficiency.

The most efficiently loaded simvastatin PLGA microspheres were embedded in
the PEG hydrogel block polymer and drilled to obtain a PolyRing. The release studies for
the simvastatin-loaded PolyRing, appeared to release simvastatin acid, the active hydroxy
form of simvastatin. On the other hand, since there was no detection of simvastatin in the
elution medium, due to the low solubility of simvastatin in the release medium, we could
not compare the release profile done by Kanjickal et al with ours.

The overall hypothesis that there would be controlled release of simvastatin from the
polymeric device, the PolyRing, was unable to be tested since there has not yet been yet a
suitable detection method for simvastatin, though there was suspected release of
simvastatin acid, its active metabolite.
CHAPTER VI
FUTURE WORK

Future work will involve the detection of simvastatin drug in the release medium. To that effect, certain suggestions including the use of a surfactant with its amphiphilic properties and protein binding methods are discussed. In recent times, the uses of surfactants have risen in connotation with hydrophobic drugs to overcome solubility limitation and to play a significant role in drug delivery systems. Studies have discovered surfactants with its amphiphilic properties aid in increasing drug solubility along with controlling the drug release rate [99,100]. Surfactants form micelles in aqueous solution lending a hydrophobic core and hydrophilic outer layer. This core assists in solubilization of the hydrophobic drug. Micelles are formed only after surfactant concentration is greater than the critical micelle concentration (cmc). Sodium dodecyl sulfate (SDS) also known as sodium lauryl sulfate (SLS) is an anionic surfactant which has a tail of 12 carbon atoms attached to sulfate group. The cmc of SDS in pure water is 0.0085 M [101] approximately 0.24% wt. Liu and Li [102] studied the controlled release of camptothecin, a hydrophobic anti cancer drug, from agarose hydrogel with the aid of SDS. The drug was encapsulated.
by the micelles and they in turn were dispersed in the gel sheet. They reported that aqueous solubility of CPT was greatly increased, for example 83 times at 1 wt.% of SDS. This method could be applied to simvastatin by similarly encapsulating the drug into an amphiphilic polymer such as SDS in order to increase solubility.

Another method to solubilize hydrophobic drugs is via protein mediated transport which is the binding of the drug to proteins acting as carriers. Carrier proteins are playing a growing role in the transportation of hydrophobic drugs through the cell membrane. The mechanism of operation follows with the drug molecule bound to a specific binding site of the protein with a binding affinity value. The transportation processes including facilitated diffusion and active transport, carry the drug to another site [103]. Hwang et al [104] observed the effect of serum proteins (α- acid glycoproteins, bovine serum albumin and calf serum) on the solubility and diffusivity of the hydrophobic drug paclitaxel. Effective solubility of paclitaxel in α- acid glycoproteins was 24.3 ± 4.0, serum albumin 64.5 ± 0.2 µg/ml and calf serum 171±9.3, a 8.1, 21.3 and 57 fold increase over that without the proteins. In a likewise approach, simvastatin could be attached to carrier proteins for which simvastatin has 95% affinity such as albumin to improve drug solubility and detection.
REFERENCES


4. de Sanctis JT. Percutaneous interventions for lower extremity peripheral vascular disease. Am Fam Physician 2001;64:1965-72


31. Levin AD, Vukmirovic N, Hwang C, Edelman ER. Specific binding to intracellular proteins determines arterial transport properties for rapamycin and paclitaxel. PNAS 2004;101:9463-9467


44. Bonetti PO, Lerman LO,Napoli C, Lerman A. Statin effects beyond lipid lowering –are they clinically relevant. Eur Heart J 2003;24:225-248


47. Liao JK. Effects of statins on 3-Hydroxy-3-methylglutaryl-CoA reductase inhibition beyond low density lipoprotein cholesterol. Am J Cardiol 2005;96:24F-33F


51. Istvan E. Statin inhibition of HMG-CoA reductase: a 3-dimensional view. Atherol Suppl 2003; 4:3-8


67. Merck & Co, Inc. Whitehouse Station, NJ. ZOCOR ® (package insert)

68. Lanjung HD, Markham A. Fluvastatin: a review of its use in lipid disorders. Drugs 1999; 57:583-606


74. Fuster V. Elucidation of the role of plaque instability and rupture in acute coronary events. Am J Cardiol 1995;76:24C-33C


84. Corsini A, Bellosta S; Baetta R. New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharma Therapeutics 1999;84:413-428


98. Ran Y, Zhao L, Xu Q, Yalkowsky SH. Solubilization of CyA. AAPS PharmSciTech, 2001;2(1), article 2

100. Torchilin VP. Structure and design of polymeric surfactant- based drug delivery systems. J Con Rel 2001;73:137-172


