SIMVASTATIN INCORPORATED PERIVASCULAR POLYMERIC CONTROLLED
DRUG DELIVERY SYSTEM FOR THE INHIBITION OF VASCULAR WALL
INTIMAL HYPERPLASIA

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SIMVASTATIN INCORPORATED PERIVASCULAR POLYMERIC CONTROLLED
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ABSTRACT

Dialysis access graft failure is a major complication in providing care to patients on hemodialysis therapy. The failure rates have been reported as high as 80% at one year for this procedure. The major cause of failure is intimal hyperplasia. Intimal hyperplasia is an exaggeration of the normal vascular wall healing response to injury resulting from the migration and proliferation of medial smooth muscle cells. To mitigate this disease condition, we developed a perivascular polymeric controlled drug delivery device which we hypothesized when applied locally, would be effective in preventing intimal hyperplasia by enabling a sustained release of the therapeutic agent to the anastamotic site. This novel polymeric device, named PolyRing, is a composite system consisting of poly (DL-lactide-co-glycolide) microspheres embedded in a poly (ethylene glycol) hydrogel. The drug for the treatment of intimal hyperplasia is encapsulated within the poly (DL-lactide-co-glycolide) microspheres. The device is snapped into place around the vessel to deliver the drug. This work focuses on evaluating the feasibility of releasing the drug Simvastatin from the device to provide localized, site specific, sustained drug delivery for the prevention of intimal hyperplasia in vascular tissue. Simvastatin, obtained in a pro-drug form, when hydrolyzed to its active form, Simvastatin Acid, acts as a potent competitive inhibitor of the 3-Hydroxy-3-methylglutaryl coenzyme A reductase. The inhibition of this enzyme suppresses the mevalonate pathway and thereby prevents the proliferation of smooth muscle cells. As the focus is on controlled localized delivery.
delivery, we hypothesize the need of using the active form as against the lactone form. Therefore, we fabricated Simvastatin and Simvastatin Acid loaded poly (DL-lactide-co-glycolide) microspheres using oil-water and water-oil-water techniques respectively. The oil-water emulsion resulted in smooth surfaced microspheres (determined by Scanning Electron Microscopy) with a particle size of approximately 50 μm (determined by dynamic light scattering), a yield range of 65-85 % and a drug loading efficiency of approximately 81%. The water-oil-water emulsion for simvastatin acid produced microparticles with a yield in the range of 60-70 % and a loading efficiency of approximately 7%. The Simvastatin Acid loaded microspheres had a smooth surface morphology with a particle size in the range of 2-25 microns. PolyRings were characterized by environmental scanning electron microscopy and revealed a uniform distribution of the drug-loaded microspheres in the poly (ethylene glycol) hydrogel matrix. The cell culture study to compare the efficacy of the two forms of the drug in inhibiting smooth muscle cell proliferation indicated that the inhibitory effect of Simvastatin Acid was significantly higher than that of Simvastatin. The release of Simvastatin Acid from the device lasted for about 70 days. Further, the Simvastatin Acid loaded device successfully inhibited the proliferation of smooth muscle cells in culture. Therefore, we conclude that the device, PolyRing, has been successfully modified for the controlled delivery of statins to inhibit intimal hyperplasia.
DEDICATION

This thesis is dedicated to god and my parents
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CHAPTER I
INTRODUCTION

Vascular access failure is a major complication in providing care to patients on hemodialysis therapy. Arteriovenous dialysis access fistulae frequently develop stenoses and occlusions at the anastomosis, commonly on the venous anastomotic side. Histologic examination of segments removed from patients with anastomotic stenosis revealed extensive intimal hyperplasia (IH) consisting of smooth muscle cells and extracellular matrix [1]. IH is also the most common basis of failure for interventional procedures like cardiac catheterization, percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass graft (CABG) surgery [2].

IH is an exaggeration of the normal vascular wall healing response to injury. It may be induced by graft compliance mismatch, flow turbulence, shear stress, vessel stretch, surgical trauma, mural ischemia, and luminal accumulation of various biochemical factors released from deposited fibrin and platelets. Migration and proliferation of vascular smooth muscle cells due to injury, inflammation and stretch are the primary causes of IH [3]. Systemic administration of anti-proliferative drugs has been largely unsuccessful in treating IH. In contrast, high concentrations of locally delivered heparin reduce neointima formation in a rabbit model of polytetrafluorethylene (PTFE) grafts [4]. The synthetic grafts, especially expanded PTFE (ePTFE) grafts have shorter maturation time periods [5]. Lin and others demonstrated that ePTFE grafts coated with
heparin significantly reduces IH formation at the anastomosis and reduces early platelet deposition in a canine femoral artery bypass model [6]. Therefore, localized delivery of therapeutic agents could be efficacious in preventing the formation of IH in hemodialysis PTFE grafts and also help reduce the systemic toxicity of certain drugs [7]. Hirko et al., [8] suggested that cyclosporine (CyA), a potent immunosuppressive agent [9], might be able to reduce the development of IH in a canine model. However, systemic administration of cyclosporine A (CyA) creates serious adverse effects. To counter the side effects of CyA, Kanjickal et al., [10, 11] developed a perivascular wrap device, in the form of a ring, named the PolyRing (patent application 10/836, 787) for the treatment of IH. PolyRing is a composite polymeric device consisting of poly (DL-lactide-co-glycolide) (PLGA) microspheres embedded in a poly (ethylene glycol) (PEG) hydrogel. The drug CyA was encapsulated within the PLGA microspheres. This ring-shaped device snugly fits around the anastomotic sites to deliver the therapeutic drugs to inhibit intimal hyperplasia.

The action of CyA for the inhibition of smooth muscle cells (SMCs) is through the immune system [8]. Statins, which have a direct route for inhibition of smooth muscle cells, were suggested for the inhibition of intimal hyperplasia. Statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are a group of cholesterol lowering drugs, which target the rate limiting enzyme in the cholesterol biosynthesis. In large scale clinical trials, statins were found to decrease the morbidity and mortality in coronary artery disease [12]. This work focuses on modifying the perivascular drug delivery system developed by Kanjickal and others by incorporating Simvastatin (SV) in it. ZOCOR®, the oral formulation of SV developed by Merck, is an inactive lactone
prodrug which is normally enzymatically converted to its active dihydroxy-open acid by intracellular enzymes [13]. Since the drug is to be delivered locally i.e., at the site of anastomosis, it is unclear whether the conversion of SV to SVA would successfully occur at the site in order for the acid form to act upon and prevent the proliferation of smooth muscle cells.

1.1 Objectives of the study

The objectives of this study, as stated above, were to modify the system developed by Kanjickal et al., by incorporating simvastatin (SV) in the PolyRing system and to further evaluate the statin incorporated device as a controlled drug delivery system. To achieve these primary objectives, studies were performed:

1. To determine surface morphologies, size distributions and loading efficiencies of the drug encapsulated microspheres. In this study, we primarily examined the effects of drug loading on the surface and size of the microparticles and further determine the efficiency of SV and SVA encapsulation in PLGA microspheres.

2. To characterize the distribution of the drug loaded PLGA microspheres in the PEG hydrogel. Here, we focus on visualizing the dispersion of microspheres in the PEG hydrogel matrix.

3. To determine the duration of drug release from sterilized and un-sterilized PolyRings under in vitro conditions.

4. To compare the inhibitory effects of the lactone form (SV) and the active form (SVA) in preventing vascular smooth muscle cell proliferation under in vitro
culture conditions. The outcome of this study would determine the type of drug to be incorporated into the PolyRing system for further studies.

5. To evaluate the efficacy of the drug loaded PolyRing device in preventing the proliferation of vascular smooth muscle cells under in vitro culture conditions.

1.2 Hypotheses

Based on the above-mentioned objectives, the following hypotheses were framed for this work.

1.2.1 Research Hypothesis

The lactone pro-drug (SV) or the active hydroxy acid (SVA) can be incorporated into the PolyRing device for sustained drug delivery applications.

1.2.2 Null Hypothesis 1

There is no significant difference in the inhibitory effects of SV and SVA in preventing the proliferation of vascular smooth muscle cells in culture i.e., there is no significant difference between the live cell counts for samples treated with SV and SVA.

1.2.3 Null Hypothesis 2

The drug loaded PolyRing system does not have a significant effect in preventing vascular smooth muscle cell proliferation in culture i.e., there is no significant difference between the live cell counts for samples treated with the drug-loaded PolyRings and non-drug loaded PolyRings.
CHAPTER II
SIGNIFICANCE AND BACKGROUND OF STUDY

The total number of vascular access procedures has reached nearly 1.5 million in 2004 with the highest costs being for graft based access (USRDS 2007). The complications arising due to vascular access has exceeded 1 billion dollars (USRDS 2005). These complications play a significant role in the hospitalizations for end stage renal disease subjects. As mentioned in the previous chapter, the primary cause of failure in dialysis access grafts is IH. Before proceeding ahead with understanding the mechanisms of IH, we first review the anatomy of the arterial wall.

2.1. Intimal Hyperplasia
The artery wall, shown in figure 2.1, consists of three distinct layers: the tunica intima, tunica media and tunica adventitia. The intima comprises a continuous endothelial monolayer on the basement membrane. The basement membrane contains type IV collagen, laminin and heparin sulphate proteoglycans, such as perlecan and syndecans. The underlying media mainly contains vascular smooth muscle cells, surrounded by their own basement membrane. They are densely packed into an interstitial matrix, which contains type I and III collagen, fibronectin and chondroitin/dermatan sulphate.
proteoglycans such as versican. The media also contains lamellae rich in the hydrophobic protein elastin. The outer adventitia contains fibroblasts in a loose connective tissue [3].

![Layers of the arterial wall](image)

Intimal hyperplasia signifies an increase in the number of cells in the intima. It is also accompanied by an increase in the amount of extracellular matrix. Physiologically, it occurs in closure of the ductus arteriosus after birth and during involution of the uterus. Pathologically, it occurs in pulmonary hypertension, atherosclerosis, and post angioplasty, in transplanted organs and in vein grafts [3]. It may also be induced by graft compliance mismatch, flow turbulence, shear stress, vessel stretch, surgical trauma, mural ischemia, and luminal accumulation of various biochemical factors released from deposited fibrin and platelets. Atherosclerosis is the most common pathological process,
which involves expansion of the intimal vascular smooth muscle cell population along with infiltration of inflammatory monocytes, T-cells, and other leukocytes from the plasma [15, 16]. The key stimuli for IH, that have been defined, are injury, inflammation, and increased mean wall stress [17].

Injury to a blood vessel occurs in two different ways: (a) mechanical injury i.e., injury inflicted by dissection of the vessel, suturing and processes like endarterectomy, thrombectomy and luminal angioplasty; (b) injury that is associated with the implantation of either autologous graft, synthetic graft or a stent. In a moderately severe injury, modeled by introducing a balloon embolectomy catheter into a rat carotid artery, the endothelium is stripped away, the vessel stretched and layers of smooth muscle cells damaged or killed [18, 19].

Once the injury occurs, an essential element in the development of intimal hyperplasia is the vascular smooth muscle cell migration and proliferation. The injury causes endothelial and smooth muscle cell (SMC) disruption and release of intracellular mitogens such as basic fibroblast growth factor (bFGF) which stimulates SMC proliferation. Following endothelial and medial injury, vascular smooth muscle cells, which generally exhibit low rates of proliferation, undergo a transformation from a quiescent, well-differentiated “contractile” state to a proliferative phenotype [20]. Such a change is generally referred to as phenotypic modulation. This transformation results in replication of the smooth muscle cells in the media followed by the migration of the cell across the internal elastic lamina into the intima. Once in the lumen of the vessel, they proliferate and eventually synthesize and secrete extracellular matrix.
Endothelial layer damage leads to the exposure of sub endothelial elements (collagen) which facilitate platelet adhesion. Platelet derived growth factor (PDGF) is released following platelet aggregation. PDGF is a chemo attractant and a mitogen for smooth muscle cells and fibroblasts [21]. Experimental evidence suggests that the chemo attractant function of PDGF plays a crucial role in the migration of the smooth muscle cell to the intimal surface [22]. In addition, the mural thrombus caused by the injury may contribute cytokines, various chemoattractants and growth factors, and angiotensin II, all of which affect the intimal thickening process.

Therefore, the migration and proliferation of vascular SMCs from the media to the intima are critical in the neointimal thickening process. In this work, we are focusing on the prevention of SMC proliferation by the sustained local delivery of statins.

2.2 Statins
Statins or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR) inhibitors, have been described as the principal and most effective class of drugs to reduce serum cholesterol levels, and have been shown to significantly reduce cardiovascular events and mortality in patients with or without coronary artery disease [12]. The different types of statins available are Atorvastatin, Cerivastatin, Lovastatin, Pravastatin and Simvastatin. This study focuses on Simvastatin (ZOCOR®) obtained as a gift from Merck.

Simvastatin is butanoic acid, 2,2-dimethyl-,1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester, [1s-[1α,3
α,7β,8β(2S*,4S*),-8αβ]]. Its molecular weight is 418.57 and the empirical formula is C_{25}H_{38}O_{5} [23]. Its structural formula is as shown in Figure 2.2.

![Structural Formula for Simvastatin (ZOCOR®)](image)

Fig. 2.2. Structural Formula for Simvastatin (ZOCOR®) [23]

In the sections to follow, we discuss in detail the action of these drugs on the cholesterol pathways. Statins exert a number of cholesterol-independent and dependant, pleiotropic effects including anti-inflammatory actions [24]. Pleiotropic effects of a drug are actions other than those for which the agent was originally developed. The action of statins on SMCs is one of the multiple effects of statins. Therefore, statins are effectively used in anti-proliferative drug therapy [12]. In section 2.2.2, this particular pleiotropic effect is emphasized, followed by the description of other significant effects.
2.2.1 Pharmological action on the Mevalonate (Cholesterol) Pathway

The action of the statins as cholesterol lowering agents is mainly due to its inhibitory effect on the mevalonate or the cholesterol pathway. These agents prevent the cholesterol formation cascade by its action on the HMGR enzyme thereby leading to reduced serum cholesterol levels. To understand its action more thoroughly, we first discuss the mevalonate pathway which leads to cholesterol formation in section 2.2.1.1. Finally, the action of statins on the above mentioned pathway would be discussed in detail in section 2.2.1.2

2.2.1.1 Biosynthesis of Cholesterol

Cholesterol is an important biological molecule that has roles in membrane structure as well as being a precursor for the synthesis of the steroid hormones and bile acids. Both dietary cholesterol and that synthesized in vivo are transported through the circulation in lipoprotein particles. Cholesterol is present in the diet of all people and is absorbed slowly from the gastrointestinal tract into the intestinal lymph. It is specifically capable of forming esters with fatty acids. About 70% of the cholesterol in the lipoproteins of the plasma is in the form of cholesterol esters. Besides the cholesterol absorbed each day from the gastrointestinal tract (exogenous cholesterol), an even greater quantity is formed in the cells of the body called endogenous cholesterol. Slightly less than half of the cholesterol in the body derives from biosynthesis in vivo. Biosynthesis in the liver accounts for approximately 10% and in the intestines approximately 15% of the amount produced each day.
An increase in the amount of cholesterol ingested each day increases the plasma concentration slightly. When cholesterol is ingested, the rising concentration of the cholesterol inhibits the most essential enzyme for endogenous synthesis of cholesterol, HMG-CoA reductase (HMGR), thus providing an intrinsic feedback control system to prevent excessive increase in plasma cholesterol concentration.

The synthesis and utilization of cholesterol must be tightly regulated in order to prevent over-accumulation and abnormal deposition within the body. Of particular importance, clinically, is the abnormal deposition of cholesterol and cholesterol-rich lipoproteins in the coronary arteries. Such deposition, eventually leading to atherosclerosis, is the leading contributory factor in diseases of the coronary arteries.

Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetate group of acetyl-CoA. The process has five major steps (as shown in figure 2.3):

1. Acetyl-CoA is converted to HMG-CoA
2. HMG-CoA is converted to mevalonate
3. Mevalonate is converted to the isoprene based molecule, isopentenyl pyrophosphate (IPP), with the concomitant loss of CO₂. IPP is converted Geranylpyrophosphate. Further, Geranylpyrophosphate (GPP) is converted to Farnesylpyrophosphate (FPP)
4. FPP to Squalene
5. Squalene is converted to Lanosterol which further yields Cholesterol.
The acetyl-CoA utilized for cholesterol biosynthesis is derived from an oxidation reaction (e.g., fatty acids or pyruvate) in the mitochondria and is transported to the cytoplasm. Acetyl-CoA can also be derived from cytoplasmic oxidation of ethanol by acetyl-CoA synthetase. All the reduction reactions of cholesterol biosynthesis use nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Acetyl-CoA units are converted to mevalonate by a series of reactions that begins with the formation of HMG-CoA. Unlike the HMG-CoA formed during ketone body synthesis in the mitochondria, this form is synthesized in the cytoplasm. However, the pathway and the necessary enzymes are the same as those in the mitochondria. Two moles of acetyl-CoA
are condensed in a reversal of the thiolase reaction, forming acetoacetyl-CoA. HMG-CoA is then obtained by the action of the enzyme HMG-CoA Synthase on acetoacetyl-CoA and a third mole of acetyl-CoA.

HMG-CoA is converted to mevalonate by HMGR (this enzyme is bound to the endoplasmic reticulum). HMG-CoA reductase requires NADPH as a cofactor and two moles of NADPH are consumed during the conversion of HMG-CoA to mevalonate. This four electron reduction of HMG-CoA to mevalonate is achieved via two successive hydride transfers in the presence of HMGR. The first reduction results in the formation of mevaldyl CoA hemi-thioacetal intermediate. The structure of the HMGR elucidates the residues that stabilize this charged intermediate. Mevaldyl-CoA subsequently decomposes to mevaldehyde and CoASH and the thiol anion is protonated by a histidine residue. In the third and final reaction, a second NADPH molecule replaces NADP+ and reduces mevaldehyde to mevalonate. A sequence comparison of the human HMGR sequence with HMGR sequences from other organisms reveals two distinct classes: eukaryotic HMGRs (class I) and prokaryotic HMGRs (class II) [26, 27].

The conversion of HMG-CoA to mevalonate is the rate limiting step of the cholesterol biosynthesis. To maintain the concentration of mevalonate-derived products, the activity of HMGR is physiologically regulated and controlled through synthesis, degradation and phosphorylation. In addition to the physiological regulation of HMGR, the human enzyme has been targeted successfully by drugs in the clinical treatment of high serum cholesterol levels [28].

Mevalonate is then activated by three successive phosphorylations, yielding 5-pyrophosphomevalonate. In addition to activating mevalonate, the phosphorylations
maintain its solubility, since otherwise it is insoluble in water. After phosphorylation, an ATP-dependent decarboxylation yields isopentenyl pyrophosphate (IPP) an activated isoprenoid molecule. IPP is in equilibrium with its isomer, dimethylallyl pyrophosphate (DMPP). One molecule of IPP condenses with one molecule of DMPP to generate geranyl pyrophosphate (GPP). GPP further condenses with another IPP molecule to yield farnesyl pyrophosphate (FPP). Finally, the NADPH-requiring enzyme, squalene synthase catalyzes the head-to-tail condensation of two molecules of FPP, yielding squalene (squalene synthase also, is tightly associated with the endoplasmic reticulum). Squalene undergoes a two step cyclization to yield lanosterol. The first reaction is catalyzed by squalene monooxygenase. This enzyme uses NADPH as a cofactor to introduce molecular oxygen as an epoxide at the 2,3 position of squalene. Through a series of 19 additional reactions, lanosterol is converted to cholesterol.

2.2.1.2 Role of Statins

All statins share a structural component that is very similar to the HMG portion of HMG-CoA, and all differ from HMG-CoA in being more bulky and more hydrophobic. The HMG like portion is covalently linked to a rigid hydrophobic group. Studies by Istvan et al. [29] showed that this moiety of statin molecules occupies the HMG binding site of the enzyme. The binding of this portion of the statin molecule is associated with a conformational change in the enzyme. This conformational change results in movement of flexible helices in the enzyme catalytic domain which exposes a binding site for the hydrophobic groups of the statins. All statins function similarly by binding to the active site of the HMGR and thus inhibiting the enzyme.
The competitive inhibitory action of statins is with respect to binding of HMG-CoA, but not with respect to binding of NADPH. The statin molecule does not occupy the NADP(H) binding pocket [25, 29]. Thus, statins sterically hinder substrate binding by binding to the active site of the HMG-CoA reductase.

2.2.2. Pleiotropic Effects of statins

Most of the pleiotropic statin actions are mediated via inhibition of the mevalonate pathway. In this way, statins not only inhibit the synthesis of cholesterol but also of a number of isoprenoid intermediates, which themselves have important biological effects. Two pleiotropic effects mediated by the inhibition of the mevalonate pathway are the upregulation of endothelial NO synthase (via inhibition of rho GTPase) and downregulation of NADPH oxidase (via inhibition of ras GTPase. Other potential beneficial effects of statins include the enhancement of nitric oxide production in vasculature and the kidneys. Statins have been shown to stabilize unstable plaques, improve vascular relaxation, and promote new vessel formation. Clinical trials and animal studies have shown that these agents reduce cardiovascular disease (CVD) risks and events, progression of nephropathy, and development of diabetes [24]. In this section we initially discuss the inhibitory effects of statins on vascular SMCs followed by other important pleiotropic effects of statins.

2.2.2.1 Effects on vascular SMC proliferation

To understand the anti-proliferative effects of statins on vascular SMCs, we first review the functioning of a smooth muscle cell followed by elucidating the action of statins on
SMCs. The cell cycle of a smooth muscle cell is depicted in figure 2.4. Growth factors derived from inflammatory cells, platelets, and the vascular wall stimulate the entry and progression of SMC into the cell cycle [30]. These growth factors which include basic fibroblast growth factor, platelet-derived growth factors (PDGF), transforming growth factor-β1, angiotensin II, and insulin-like growth factors utilize distinct signaling pathways, but do converge upon common regulators of the cell cycle, namely, the cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors [31]. These in turn are regulated by the family of Cdk inhibitor proteins, such as p16, p21Waf1 and p27Kip1 [32].

![Cell cycle of a Smooth Muscle Cell]

The cell cycle begins with the G1 phase. It then passes on to the S (DNA synthesis) phase via the restriction site R. From the DNA synthesis phase, it goes on to
mitosis (M). 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 [33]. Hyperphosphorylation of the retinoblastoma gene product (Rb), which functions as a molecular switch dedicating the cell to DNA replication, results in the release of the E2F, which induces the expression of the genes required for the progression through S, G2 and M phases [32].

FPP and GGPP are important lipid attachments for the post-transitional modification of a variety of proteins, including Ras and Rho GTP-binding proteins. The isoprenoid, geranylgeranyl, and the protein that it post-translationally modifies (Rho) mediates PDGF-induced cell cycle progression by down-regulating the expression of the Cdk inhibitor p27Kip1 and stimulating the activity of Cdk-2 and hyperphosphorylation of Rb [34].

The inhibition of the HMGR by statins thereby prevents the formation of mevalonate. This further reduces the catabolism of the isoprenoid intermediates of the cholesterol pathway such as FPP and GGPP. As mentioned above, GGPP post-translationally modifies the Rho protein responsible for mediating the PDGF induced cell cycle progression. Thus, treatment with statins decreases the Rho geranylgeranylation, inhibits Cdk activity and Rb hyperphosphorylation, and prevents SMC synthesis (as shown in figure 2.5).
Fig. 2.5. Cholesterol and SMC inhibition cascade by statins
Raiteri et al., [35] demonstrated that statins (Simvastatin, Cerivastatin and Fluvastatin) dose-dependently decreased the smooth muscle cell proliferation, independently of their ability to reduce plasma cholesterol. The delay of cycling cells in the G1 and G2/M phases leads to apoptosis in different smooth muscle cells [36, 37]. In a study done by Indolfi and others [38], Simvastatin inhibited vascular smooth muscle cell proliferation in vitro and further reduces neointimal formation in a rat model of vascular injury. Erl et al., [39] found that the statins induced cell death preferentially in the neointimal smooth muscle cells, thereby, suggesting that statins enhance the rate of apoptosis in the cells of the neointimal origin.

2.2.2.2 Effect on Chronic Heart Failure
Statin therapy decreases myocardial rac1-GTPase activity and inhibits upregulated NADPHoxidase associated with increased rac1 activity in patients with coronary artery disease (CAD) and in cases with failing myocardium respectively. These findings suggest that statins have beneficial effects on symptoms of heart failure irrespective of its cholesterol lowering effects [24, 40]

2.2.2.3 Effects on Inflammation
The vascular inflammatory response is a complex process that leads to thrombus formation, angiogenesis, neointimal thickening, and atherosclerosis. Atherosclerotic lesions arise due to an interaction between four major cell types: endothelial cells, smooth muscle cells, macrophages, and lymphocytes. Statins may interfere directly with several key mechanisms necessary for involvement of the different cellular elements in the
inflammatory response in all the steps of atherogenesis. High plasma levels of markers like P-selectin, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), soluble intercellular adhesion molecule-1, monocyte-chemotactic protein-1 (MCP-1), and C-reactive protein (CRP), which are constituents of the inflammatory cascade, are shown to play a role in plaque rupture. It has been found that Pravastatin lowers CRP levels thereby eliminating the cardiovascular risks associated with it. Pravastatin has increased the incidence of transplant acceptance (heart and kidney) by reducing the cytotoxicity levels of the natural killer cells in the recipients. Statins may also affect thrombus formation and levels of plasminogen activator inhibitor-1 and fibrinogen [24, 41].

2.2.2.4 Statin interactions with Monocyte/Macrophage/T-cells and effects on atherogenesis

Patients with hypercholesterolemia have an increased adhesion of monocytes to endothelium. An abnormal monocyte function is responsible for this phenomenon of increased adhesion. Statins reduce monocyte infiltration and proliferation in rats with coronary inflammation. Statins, Simvastatin in particular, attenuates endothelial adhesion molecule and monocyte CD11b expression. Statins inhibit the expression of the major histocompatibility complex - II (MHC-II) by IFN gamma and therefore suppress the MHC-II mediated T-cell activation. The inhibitory effect of statins on several important proinflammatory cytokines results in altering the function of Th1 (T-helper) cells. This alteration of the Th1 cells has a significant therapeutic effect. This is because Th1 cells play an important role in mediating several auto-immune diseases like multiple sclerosis, rheumatoid arthritis, systemic lupus, and type 1 diabetes [24, 40]. These observations
signify the importance of statins in attenuating the inflammatory process and its effect on CVD risk reduction.

2.2.2.5 Effects on Oxidative Stress

A healthy endothelium plays a central role in cardiovascular control. An altering balance between relaxing and contracting factors, procoagulant and anticoagulant substances, and between pro-inflammatory and anti-inflammatory mediators results in endothelial dysfunction (ED). ED may play a significant role in the pathogenesis of atherosclerosis. Increased oxidative stress and inflammation are different risk factors that cause ED. An imbalance is the relative concentrations of oxidants and antioxidants results in oxidative stress. Statins as potent antioxidants and anti-atherosclerotic agents are attractive therapeutic options for preserving normal vascular function and blood flow. Various statins have been shown to inhibit the uptake and generation of Ox-LDL which is deleterious to endothelial and vascular smooth muscle cells. The inhibitory action of statins on oxidative stress and smooth muscle cell migration has been considered an additional basis for anti-atherosclerotic therapy. When added to SMC cultures, these HMG-CoA reductase inhibitors resulted in approximately 50% suppression of human coronary SMC migration [24].

2.2.2.6 Effects on Thrombosis

Statins are considered to play a pivotal role in modulating the levels of important elements in the process of thrombosis. Statins have varying effects on
different prothrombotic factors, such as tissue factor pathway inhibitor, tissue factor, platelet aggregation, blood and plasma viscosity, fibrinogen and plasminogen activator inhibitor 1 (PAI-1). Long-term statin treatment leads to a significant reduction of fibrinogen levels, normalization in thrombin generation, and a reduction of platelet aggregation induced by ADP in hypercholesterolemic patients. Both membrane-bound and soluble CD40L (sCD40L) may interact with CD40 expressed on vascular cells, resulting in inflammatory and prothrombotic responses. Thus, CD40L-CD40 interaction could be the mechanism that links inflammation and the prothrombotic state in hypercholesterolemia. OxLDL may promote the CD40-CD40L expression in human atheroma. Statins may act by limiting the expression of this receptor-ligand complex either directly or through reduced lipoprotein levels [24, 42].

2.2.2.7 Effects on Platelet Function
Statins play a significant role on platelet functions and levels of adhesion molecules by significantly reducing P selectin or ICAM-1 levels. ADP from activated platelets is a potent stimulator of oxygen-free radical release from neutrophils. Statins inhibit platelet ADP and ATP release and the consecutive augmentation of neutrophil oxygen-free radical release. Thus, statins may affect platelet-neutrophil interactions [24].

2.2.2.8 Effects on Vasculogenesis
Statins mediate neovascularization, i.e., vasculogenesis and contribute to the reduction in recurrent cardiovascular disease events. Statins have shown to mobilize endothelial
progenitor cells (EPCs) from the bone marrow which plays a role in maintaining vasculogenesis. These EPCs are seen immediately after a coronary event and line the endothelium of myocardial vessels. Statins also lower the plasma level of vascular endothelial growth factor (VEGF) in coronary artery disease (CAD) patients [24].

2.2.2.9 Effects on Re-endothelialization after Balloon Injury

Re-endothelialization at sites of spontaneous disruption results from the migration and proliferation of endothelial cells from viable endothelium adjacent to the site of injury. In an animal model (rat), statin treatment accelerated re-endothelialization of the balloon-injured arterial segments and resulted in a dose-dependent significant reduction in neointimal thickening. The protein Rho activates signals that regulate apoptosis and statins prevent the isoprenylation of this protein. Statin treatment is associated with reduced cellular proliferation, leukocyte accumulation, and increased apoptosis after injury [24].

2.2.2.10 Effects on Endothelial Function

The vascular endothelium serves as an important autocrine/paracrine organ that regulates vascular wall contractile state and cellular composition. Hypercholesterolemia impairs the effective functioning of the endothelium i.e., leading to ED. An important characteristic of the ED is the impaired synthesis, release, and activity of endothelial-derived nitric oxide (NO). Endothelial NO inhibits several components of the atherogenic process. It mediates vascular relaxation and inhibits platelet aggregation, vascular smooth muscle proliferation and endothelial-leukocyte interactions. Statins, by lowering serum
cholesterol levels, improve the functioning of the endothelium, vasodilation in particular.
Statins upregulate endothelial nitric oxide synthase (eNOS) expression and activity and
reverse the downregulation of eNOS expression by hypoxia and oxidized low-density
lipoprotein (ox-LDL) under cholesterol-clamped conditions. Statins upregulate eNOS
expression by inhibiting Rho isoprenylation. Statins also have a direct effect on
endothelin-1 (ET-1) production as observed by a decrease in its basal transcription rate of
the bovine aortic endothelial cells. ET-1 is a powerful vasoconstrictor and decreasing ET-
1 levels potentially reduces vascular resistance and improves blood flow in coronary and
systemic circulation [41].

2.2.2.11 Effect on Atherosclerotic Plaques
Disruption of unstable atherosclerotic plaques is seen as the main causes in acute
coronary events. Disruption of a formed plaque is a complex pathologic process that is
central to the initiation of the acute coronary syndromes. Local thrombosis occurring
after plaque disruption results from complex interactions among the lipid core, smooth-
muscle cells, macrophages, and collagen. Most vulnerable atherosclerotic lesions occur in
moderately stenotic vessels and are characterized by a lipid-rich core and excess activated
inflammatory cells. Macrophages release matrix metalloproteases that degrade plaque
matrix connective tissue, weaken the fibrous cap, and render them susceptible for rupture.
Statins have been shown to decrease the levels of metalloproteases, oxidized-LDL (ox-
LDL), core lipid content, and macrophages and to increase collagen content in plaque
matrix, actions that increase plaque stability [24, 43]
2.2.2.12 Effects on Renal Function

Renal injury initiates inflammatory cascades that involve similar cellular events as seen in vascular tissue. Statins inhibit events in the process that alter the pathway of renal injury. Statins ameliorates the structural and functional changes of diabetic nephropathy independently of serum lipid levels in hyperglycemic insulin deficient diabetic rats. Similar effects were observed in obese insulin resistant rats with glomerular disease. Statins decrease systolic blood pressure, albuminuria, and cortical necrosis in animal models of renal injury. Statins inhibit mesangial cell proliferation and monocyte/macrophage infiltration in a rat model with glomerular nephritis. Thus statins attenuate renal injury in both in vitro and in vivo studies [24, 43].

2.2.2.13 Effects on Glucose Metabolism

Type II diabetes is a condition where the target cells fail to respond to ordinary levels of circulating insulin. Statins modulate insulin activated signaling cascades that mediate glucose uptake. Insulin increases skeletal muscle perfusion and substrate delivery by enhancing eNOS activity. Statins also increase eNOS expression, which may result in increased capillary recruitment and glucose disposal. Diabetes mellitus aggravates ischemic damage in a rat skeletal muscle ischemia-reperfusion injury model. A 6-week therapy with statins resulted in a reduction of the lower limb ischemia [24, 44].

2.2.2.14 Effects on Bone Metabolism

A dynamic balance between bone formation and bone resorption is critical to maintaining healthy bones. An imbalance arises when resorption exceeds bone formation resulting in
diseases of bone metabolism like postmenopausal osteoporosis. Antiresorptive bisphosphonate drugs containing nitrogen inhibit bone resorption by preventing protein prenylation in osteoclasts. This mechanism of action could also indicate possible effects of statins on bone remodeling. Murine osteoclast formation in cultures was inhibited by statins and alendronate, a nitrogen-containing bisphosphonate. The interaction between bone-forming osteoblasts and bone-resorbing osteoclasts is very close. Osteoblasts arising from the mesenchymal stem cell precursors differentiate in response to factors like bone morphogenetic proteins (BMPs) and vascular endothelial growth factors (VEGF). Osteoblasts are proximal to endothelial cells and vascular invasion is a prerequisite for endothelial bone formation and fracture healing. Statins stimulate VEGF expression in osteoblasts via reduced protein prenylation and the phosphatidylinositol-3 kinase pathway, thereby promoting osteoblastic differentiation. Furthermore, rabbit osteoclast formation and activity are inhibited by statins and by alendronate. These findings suggest that statins may act on osteoclasts and possibly may inhibit bone resorption. Statins have been found to increase the number of osteoblasts and the amount of new bone formation in mouse skull bones in vitro. Similar effects were also seen in vivo by injecting the drug into the skull bone of mice. Also, oral administration of statins to rats increased trabecular bone volume and the rate of new bone formation [24, 45]

2.2.2.15 Effects on Cognitive Function

Cognitive impairment in elderly people is known to be a heterogeneous condition that in most cases has pathological features consistent with Alzheimer's disease. Changing the plasma cholesterol concentration may alter input of sterol into the CNS, statins cross the
blood-brain barrier and partially inhibit the rate of cholesterol synthesis in the brain and finally deleting the 24-hydroxylase in the brain partially blocks cholesterol excretion from the CNS. In a large observational study, treatment of statins was associated with a 60% to 73% lower prevalence rate of Alzheimer's disease [24].

2.2.2.16 Oncoprotective Effects

Experimental evidence suggests that statins may also have oncoprotective effects by inducing certain tumor cell types, such as acute myelogenous leukemia, to undergo apoptosis in a sensitive and specific manner. Anti-tumor impact of statins compounds were demonstrated in several experimental animal studies [24].

2.2.3.17 Effect on Ischemic Stroke

Ischemic stroke is the third leading cause of death in the United States and is a major cause of long-term disability, especially in the elderly population and in patients undergoing cardiovascular surgery. Statins are beneficial for ischemic strokes which can be attributed to the effects of the drug on endothelial function and the vascular wall mentioned previously. Cerebral vascular tone and blood flow are regulated by endothelium-derived NO. Mutant mice lacking eNOS have shown to develop larger cerebral infarcts following cerebrovascular occlusion. The upregulation of eNOS accounts for most of the neuroprotective effects of these agents. Statins have shown to attenuate P-selectin expression and leukocyte adhesion via an increase in NO production in a model of cardiac ischemia and reperfusion. Thus, the beneficial effects of statins in
an ischemic stroke are primarily due to their ability to upregulate eNOS expression and activity. [41].

2.3 Drug Delivery Systems (DDS)

The design of a drug delivery system focuses on the controlled delivery of the therapeutic agent to its site of action. Other advantages of using controlled-delivery systems can include the maintenance of drug levels within a desired range, the need for fewer administrations, optimal use of the drug, and increased patient compliance. The new drug dosage forms aim at improving the access of active molecules to the action site in the organism by carriers developed to deliver and to protect the active molecule towards the biological target and designing a sustained-release drug formulation to provide a long-lasting effect with a slow release of the active molecule [46]. The idea behind controlling the drug delivery is to achieve more effective therapies while eliminating the potential for both under- and overdosing. This could be explained by analyzing the changes in the blood plasma level after administering a dose of therapeutic agents using the means of injections or traditional tablets. The drug level in the blood follows the profile shown in Figure 2.6, in which the level rises after each administration of the drug and then decreases until the next administration. The key point with traditional drug administration is that the blood level of the agent should remain between a maximum value, which may represent a toxic level, and a minimum value, below which the drug is no longer effective. In controlled drug delivery systems designed for long-term administration, the drug level in the blood follows the profile shown in Figure 2.7, remaining constant, between the desired maximum and minimum, for an extended period of time. The drug
delivery systems formulated to maintain a desired blood plasma level between the toxic levels and minimum effective levels for prolonged durations are called zero-order systems since the release of the drug is at a constant rate [47]

Fig. 2.6. Drug concentration following single drug dosage (injection or tablets) as a function of time

Fig. 2.7. Drug concentration following single drug dosage (controlled release) as a function of time
2.3.1 Vascular Drug Delivery

The concept of localized sustained drug delivery would help the cause of restenosis. Due to the focal nature of an atherosclerotic disease, the efficacious method would be to locally deliver the drug to the diseased artery. Conceptually, local drug delivery might also help reduce the toxic side effects of potent drugs by a decrease in magnitude (of multiple orders) the general drug dosage which in turn will lower the drug’s exposure to circulation. Incorporation of the drug into a degradable polymeric matrix results in sustained release of the drug thereby prolonging the duration of treatment. Sustained release is important in cases of chemically unstable drugs which would rapidly degrade. Moreover, different drug release patterns could be achieved by varying polymer class and compositions. There are two generic approaches in vascular local drug delivery. Namely, a drug can be delivered to an injured arterial segment from an adventitial aspect of the vessel or it can be applied intraluminally i.e., from inside the artery [48].

2.3.1.1 Intraluminal Delivery

In general, an intraluminal stent-graft device consists of a supporting frame covered with a synthetic graft material. The main advantage of intraluminal drug delivery is that it is performed simultaneously with a vascular intervention. The procedure of delivery is noninvasive as the access is through a percutaneous approach. The medication is distributed primarily in the intima and is therefore effective in treating lumen dependent processes, such as thrombosis. Partial redistribution to the adventitia through the vasa vasorum occurs after a while. This mode of delivery is an attractive option but the
implantation of these devices is not desirable in all cases. This is because; these stents often impose several requirements on the drug, vehicle and the delivery system. A diseased vessel wall possesses prothrombotic properties and therefore, the applied drug should not further increase local thrombogenicity. An issue of biocompatibility and biodegradability of the delivery system arises due to the long-term presence of a foreign material in immediate contact with the components of the artery. Leaching of the delivered dose of drug to the intimal layer is very intensive because of the continuous blood flow through the treated arterial segment. Finally, specialized delivery catheters or endoluminal stents and other equipments are required for the application and monitoring of these devices [48, 49].

Desired characteristics of the stent grafts are adequate flexibility, kink resistance, longitudinal strength, ease of deployment and reliable fixation system. In case of stents for abdominal aortic aneurysms (AAA), persistant blood flow into the aneurysm following stent grafting, termed endoleak, causes pressurization of the aneurysm sac indicating a failed procedure and risk for AAA rupture. Endoleaks are due to incomplete sealing, or exclusion of the aneurysm sac or vessel, and reflux of blood flow into the sac. Four types of endoleaks are currently recognized, namely: [50]

*Type-I endoleak:* Occurs due to the presence of a persistant channel of blood flow which develops due to inadequate or ineffective seal at the graft ends. This type is usually present early in the course of the treatment but may also occur when blood erodes through a blood-clot seal around the area of the device fixation to the vessel wall.

*Type-II endoleak:* is a retrograde type of endoleak. It occurs when there is a persistant blood flow into the aneurysm sac due to retrograde blood flow from other arteries.
Type-III endoleak: is due to inadequate or ineffective seal at the graft joints or due to rupture of the graft fabric. This type of endoleak develops early due to technical issues or late due to device breakdown.

Type-IV endoleak: is related to the porosity and passage of blood through the fabric of the graft.

2.3.1.2 Perivascular Delivery

The rationale behind perivascular delivery is to provide a local depot of the drug proximal to the adventitia of the blood vessel. Perivascular drug release strategies offer a physiologic advantage in comparison with systemic modes of administration in terms of attaining a localized sustained release and direct drug delivery and deposition on the adventitial aspect of the artery. The drug passively diffuses into the vessel wall driven by a concentration gradient. The vasa vasorum system in the adventitia further facilitates the diffusion process. It consists of a network of arterioles, capillaries and venules which nourish the components of the vessel wall. The vasa vasorum in atherosclerotic plaque is more extensive than in normal blood vessels and deeply penetrates the vessel wall. On exposure to the vasa vasorum, the drug gets evenly distributed in the whole arterial segment, including the inner most layers of the media and intima [48].

2.3.1.2.1 Perivascular drug release uptake

The drug’s metabolic pathway and transport processes are critical factors in understanding and further, optimizing the drug release from a perivascular delivery system. Lovich et al. [51] studied the pharmacokinetic aspects of perivascular delivery.
They attempted to characterize the pathways of drug clearance from the perivascular space and subsequent drug deposition in the different layers of the blood vessel wall. This study describes a methodology that differentiates between local drug delivery from the perivascular drug depot and a secondary drug influx into the targeted arterial site through systemic circulation. Drug clearance from the perivascular space can occur primarily through transmural diffusion (Figure 2.8, pathway a) and/or absorption by extraarterial capillaries (Figure 2.8, pathway b).

![Drug clearance routes from perivascular space](image)

Fig. 2.8. Drug clearance routes from perivascular space. (a) transarterial transport i.e., entry into the lumen and (b) absorption by extra arterial capillaries [51]

The drug (heparin) deposition was measured, firstly, in a native state and secondly, by eliminating pathways that could lead to endovascular administration from the systemic circulation (Figure 2.9, pathway c), extra arterial capillary uptake (Figure...
2.9, pathway d) and/or direct diffusion from the perivascular space (Figure 2.9, pathway e).

![Diagram](image)

Fig. 2.9. Drug entry and deposition from the perivascular space. (c) endovascular administration; (d) after absorption by extra arterial capillaries; (e) direct diffusion [51]

Lymphatic vessels and capillaries of the skeletal muscle or other tissues constitute the extra arterial microvessels. In this study, these pathways were analyzed by measuring the clearance of inulin which was systemically administered. Along with inulin, heparin was applied perivascularly to observe the alteration of urinary excretion and arterial deposition due to systematic elimination of pathways of vessel wall clearance and entry. The following observations were made:

i) The occlusion of the blood vessel did not effectively decrease the inulin excretion or the heparin deposition.
ii) The silicon wraps used to prevent exposure to the extra arterial capillaries reduced the inulin excretion but did not alter the heparin deposition. A significant observation was that the amount of heparin deposited after perivascular delivery was higher than intraperitoneal administration.

The inference from the above observations is that majority of the perivascularly released drug is absorbed by the extra arterial microvessels but virtually all the drug deposited in the arterial walls is a result of direct diffusion of the drug from the perivascular space into the three layers of the arterial wall. The phenomenon of drug absorption into the extra arterial space is due to two factors namely, 1) higher diffusive resistance of the artery than that of a capillary and 2) greater total surface area of absorption of the capillaries in comparison to that of an artery. Furthermore, in large and thick arteries, the transvascular resistance (resistance in the vessel wall) is greater. This would further increase the drug absorption by the extra arterial capillaries. These two factors facilitate the partitioning of the drug to the extra arterial space. The presence of a large transmural concentration gradient enhances the drug diffusion into the vessel wall from the perivascular space. Therefore, the drug deposition in the walls of the artery is due to direct diffusion from the perivascular space itself. An important factor that would aid this deposition is the impact of the vasa vasorum. Large arteries are highly vascularized by the vasa vasorum which absorbs the drug and helps transport it deep within the vessel walls into the media and intima, thus enhancing transmural clearance.

Therefore, a balance between these factors, high transvascular resistance and contributions of the vasa vasorum, is important in determining the drug pharmacokinetics within the vessel wall. Thus, the perivascular localized delivery of therapeutic agents
would lead to drug deposition in the arterial wall due to the extensive vasa vasorum network that aids in drug absorption and transportation.

In hemodialysis access grafts, IH can develop in the graft and in the native vessels around the anastomoses. Therefore, drug transports via the transgraft, transarterial, and transvenous pathways (Figure 2.10) are all potentially important in perivascular drug therapies addressing the concern of IH in dialysis access graft.

Fig.2.10. Perivascular pathways for drug transport [7]

In a study by Goteti et al., [7], the drug concentrations and diffusivities of the antiproliferative drug, dipyridamole, applied perivascularly, in hemodialysis vascular grafts and the surrounding native vessels was evaluated. They reported higher diffusivities of dipyridamole through native arteries and veins than the explanted graft,
probably because the drug has to traverse the adventitial tissue layer surrounding the graft and the graft wall following perivascular drug administration. They suggested that finding might be advantageous for the transgraft pathway as IH occurs more frequently in the native vessels around the anastamosis than in the grafts [52]. This adventitial tissue layer surrounding grafts acts as an additional barrier to drug transport via transgraft pathway and would prevent any drug loss via this pathway. This would facilitate drug to be more available via transarterial or transvenous pathway, where hyperplasia actually originates.

2.3.2.1.2 Current studies justifying the perivascular release of drugs

Localized controlled drug delivery can be achieved through endovascular applications (catheter-mediated) or through implanted perivascular drug release systems. Perivascular systems offer generally large loading capacities, high reproducibility of release profiles and are relatively simple [11].

Edelman and his coworkers carried out studies with heparin to investigate the effectiveness of perivascular or adventitial delivery. In their work, they observed that heparin was ineffective when administered intravenously or subcutaneously. Further, they reported that heparin delivered from perivascularly placed ethylene-vinyl acetate copolymer (EVAc) matrices suppressed smooth muscle cell proliferation and reduced the luminal occlusion of the injured artery to 27.1% [53]. In accordance with the above work, Edelman also observed that stainless steel stents, coated with an inert polymer, releasing heparin, increased intimal hyperplasia due to the development of an inflammatory reaction to the polymer material-heparin complex. Therefore, these
findings led to the conclusion that perivascular administration could be used to deliver drugs which are potentially potent but discarded as inhibitors of SMC proliferation due to their ineffectiveness when administered systemically. Periadventitial delivery could also be used to deliver agents which are unstable in solution, are of low solubility, and have rapid degradation characteristics [53]. Further, Creel and others delivered paclitaxel, a hydrophobic drug, through endovascular and perivascular application to observe the differences in the distribution and deposition of the drug. They found elevated drug concentration levels in the intima with endovascular application and in the adventitia with perivascular delivery [54].

Drug eluting stents are effective in coronary arteries owing to the fact that intimal hyperplasia in the coronary artery is a secondary lesion resulting from balloon angioplasty. However, in the case of dialysis access grafts and other peripheral revascularization procedures, intimal hyperplasia is the primary lesion. Therefore, angioplasty and stent placement are not necessary [55]. Detailed studies with stents coated with labeled heparin show a significant loss of the drug to the systemic circulation [56].

Mishaly et al [57] used EVA (Ethylene-vinyl acetate co-polymer) matrices as drug platforms for controlled release of Colchicine. Significant inhibition of neointimal proliferation after balloon injury was obtained at a dosage of 2µg/day delivered from the EVA perivascular matrices. The shape of the polymer matrix was rectangular and one surface was sealed with blank EVA film to obtain unidirectional release of the drug.

Fishbein et al [58] assessed the antiproliferative effects of PDGFR-β-specific tyrphostin AG-1295 in vitro and in vivo using a site specific controlled delivery system
made of EVA (Ethylene-vinyl acetate co-polymer). Tyrphostins are low molecular weight inhibitors of protein tyrosine kinases. They observed a significant antirestenotic effect of Tyrphostins in a rat carotid model and a marked antiproliferative activity of AG-1295 to the rat vascular smooth muscle cells in culture. Later, Fishbein and co-workers used a blend of EVA (Ethylene-vinyl acetate co-polymer) and PEG perivascular polymer matrices to release Mithramycin in balloon injured rat carotid arteries. Mithramycin potently inhibits transcriptional initiation from P1 and P2 promoters which are the promoter sites for c-myc gene. Proliferation of smooth muscle cells (SMC) is thereby inhibited by the downregulation of c-myc expression. A rapid and a controlled release of mithramycin was observed from EVA/PEG matrices in comparison with the release from plain EVA matrices both in vitro and in vivo. A 50% reduction in neointimal formation was observed with the drug-loaded EVA-PEG matrices [59]. However, EVA is hydrophobic, non-compliant, nondeformable and non-degradable under physiological conditions [60].

Masaki et al [55] examined the feasibility of using an injectable system (ReGel®) to inhibit neointimal hyperplasia of arteriovenous hemodialysis grafts. The drug used for inhibition was Paclitaxel. The delivery platform i.e., ReGel® is a triblock copolymer of polylactide-co-glycolide-polyethylene glycol- polylactide-co-glycolide. The temperature dependent reversible transition between the liquid and gel phase enables it to be injectable.

In a study by Edelman et al [60] heparin releasing from the alginate films inhibited growth of bovine vascular smooth muscle cells in tissue culture in a dose-dependant manner. The drug delivery system consists of Heparin encapsulated
poly(lactic-co-glycolic acid) (PLGA) microspheres sequestered in a calcium alginate gel. It substantially reduced intimal hyperplasia without causing any bleeding as compared to that caused by ethylene-vinyl acetate co-polymer (EVA) matrix-heparin releasing devices.

Jackson et al [61] studied the release of paclitaxel from poly (lactic-co-glycolic acid) (PLGA) films. PLGA was blended with biocompatible plasticizers like MePEG (Methoxypolyethylene glycol) or a diblock co-polymer composed of poly (D, L-lactic acid)-block-methoxypolyethylene glycol (PDLLA-MePEG). Paclitaxel release from the PLGA/MePEG films was very low with 5% of the encapsulated drug being released over two weeks. The addition of the 30% diblock to paclitaxel loaded PLGA resulted in a substantial increase in the release rate of paclitaxel. The paclitaxel loaded films degraded after 28 days in rats.

Pires et al [62] developed perivascular cuffs constructed out of poly (ε-caprolactone) (PCL) formulation to induce restenosis. The cuffs (a longitudinal cut cylinder with internal diameter of 0.5 mm and an external diameter of 1 mm) were then loaded with antirestenotic compounds like Paclitaxel and Rapamycin and placed around the femoral artery of mice in vivo. This resulted in a perivascular sustained release, restricted to the cuffed vessel segment with no systemic adverse effects or effects on contra lateral femoral arteries and caused a reduction in intimal thickening by 76% (for Paclitaxel) and 75% (for Rapamycin).

Ark Therapeutics Ltd. is developing a vascular endothelial growth factor D (VEGF-D) gene in an adenoviral vector, delivered locally to the adventitial surface of a graft-vein anastomosis by means of a collagen collar device. This device named Trinam®
would, thereby, be used in the treatment of intimal hyperplasia. Adventitial delivery of VEGF using a silastic collar as a gene delivery reservoir prevented smooth muscle cell proliferation without evidence of new blood vessel formation was done on an in vivo rabbit model of intimal thickening in carotid arteries, indicating that the mechanism by which VEGF inhibited intimal hyperplasia did not involve angiogenesis [63]. The device has received Orphan Drug Status in the US and Europe and is presently in its Phase II/III development [64].

2.4. PolyRing - Perivascular Polymeric Controlled Drug Delivery System

Kanjickal et al [10, 11] developed a localized perivascular drug delivery system for the prevention of intimal hyperplasia. This device, named PolyRing, incorporated Cyclosporine A, an immunosuppressive agent, for inhibiting the proliferation of smooth muscle cells.

![Fig.2.11. Idealized illustration of the PolyRing system [10]]
PolyRing, shown in Figure 2.11, consists of PLGA microspheres (which carry the drug) dispersed or embedded in a PEG hydrogel block. The device is a cylindrical ring structure with an axial slit which enables it to snugly fit onto the artery as well as the graft in the vicinity of the anastomosis. The ring is made secure by matching the size of the ring with the diameter of the blood vessel. The drug, which is encapsulated within the PLGA microspheres, releases through the device over an extended period of time thus inhibiting the proliferation of the vascular smooth muscle cells.

The PolyRing provides flexibility in terms of the type of drug that can be delivered and the duration of drug release that needs to be achieved. PLGA microspheres have been shown to encapsulate a wide range of therapeutic agents with release lasting from a few days to several months. These devices are not drug specific and also have the potential to achieve combination therapy. The EVA sheets developed by Mishaly and Fishbein are fabricated by dissolving Colchicine/AG1295 and EVA in methylene chloride [57, 58]. Therefore, the release characteristics cannot be modified or controlled to a great extent. However, the release from the PLGA microspheres in the PolyRing could be modified or specifically controlled by increasing the number and size of microspheres. The perivascular sheet consisting of heparin-encapsulated PLGA micropsheres sequestered in an alginate gel developed by Edelman could achieve release up to 40-42 days. However, this sheet requires mechanical support to stay fixed around the anastomotic site [60]. The PLGA films developed by Jackson were sutured in place to immobilize the film [61]. The design of the PolyRing is such that no additional retention devices will be needed, as compared to other polymeric sheets, to keep the ring in close contact with vessel adventitia.
CHAPTER III
MATERIALS AND METHODS

3.1 Materials
Poly (ethylene glycol) (PEG) of 3350 molecular weight was bought from Sigma (St. Louis, MO, USA). Dichloromethane (DCM) and Acetonitrile (ACN) were purchased from Aldrich (Milwaukee, WI, USA) and used as received, without further purification. Simvastatin, ZOCOR®, was a gift from Merck (West Point, PA) and poly (DL-lactide-co-glycolide) (PLGA) 85/15 (inherent (inh.) viscosity: 0.78 dl/g) was obtained from Absorbable Polymers International. Poly (vinyl alchohol) (PVA) was purchased from Sigma Aldrich. Triphenyl methane triisocyanate (Desmodur RE) was received as a gift from Bayer (Pittsburg, PA, USA). Phosphate buffered saline (PBS) were obtained in the powder form from Aldrich (Milwaukee, WI, USA), and was dissolved in 1 L of distilled-deionized water to yield a concentration of 0.01 M PBS.

3.2 Methods
The lactone and the active forms of the drug i.e., SV and SVA were encapsulated in PLGA microparticles using modified oil-water (o/w) and water-oil-water (w/o/w) techniques respectively.
3.2.1 Preparation of Simvastatin loaded PLGA microspheres

Microspheres were prepared by a standard solvent evaporation o/w (single emulsion) method as described elsewhere [65]. Briefly, 1g of PLGA and a known amount of the drug (SV), according to the theoretical loading value of either 0.1 or 0.2, expressed as (weight of drug (mg)/weight of polymer (mg)), were dissolved in 10 ml of DCM to constitute the organic phase. This solution was further emulsified, at room temperature, in 50 ml of an aqueous PVA solution (5%) using a stirring motor with an impeller (LR400D, Yamato Scientific Co Ltd) for 10 minutes in a two-necked round bottom flask at a constant speed of 350 rpm. The organic solvent was permitted to evaporate under atmospheric conditions on a lab stirrer (Fisher Scientific) for 5 hours leading to the hardening of the initially soft and viscous microparticle droplets. The microparticles were further collected by centrifugation at 2500 rpm for 10 minutes (Sorvall), washed thoroughly with distilled-deionized water three times, shell frozen and lyophilized (Labconco) for complete removal of water from the system and the final product was obtained in the form of a free flowing powder of PLGA microspheres loaded with SV. Blank PLGA microspheres, with no drug in them, were prepared in a similar manner as described above without the drug formulation being added in the organic phase. The schematic for the single emulsion technique is as shown in figure 3.1.
3.2.2. Preparation of Simvastatin Acid loaded PLGA microspheres

As mentioned in the introduction and background of study section, SV is a lactone prodrug, which is activated to its β-hydroxy acid form (SVA) by hydrolysis in vivo. Therefore, we converted the lactone form to the acid form according to the protocol developed by Merck.

3.2.2.1 Conversion of Simvastatin to Simvastatin Acid

To open the lactone ring in the statin, 8 mg of SV (0.019 mMol) was dissolved in 0.2 ml of ethanol (95-100%) and then 0.3 ml of 0.1 N NaOH was added. After heating at 50°C for 2 hours, the resulting solution was neutralized with HCl to a pH of approximately 7.2 and the volume was brought up to 1 ml with distilled water. A large volume of stock solution was prepared which was used for all the studies. The solution was lyophilized to obtain SVA in a free-flowing powder form.
2.2.2 Fourier Transform Infrared Spectroscopy (FTIR)

To verify the conversion of SV to SVA, an FTIR analysis using the Nicolet™ Nexus™ FT – IR Spectrometer was performed and the spectra obtained were analyzed for conversion using the OMNIC computer based software (version 6.0 a). Neat samples of SV and SVA were placed on a sample holder (Foundation Series, ThermoSpectra-Tech) for the analysis. The number of scans performed was sixteen.

In the electromagnetic spectrum, the section between the visible and microwave regions is broadly classified as the infrared region. This portion of the spectrum is further divided into three regions namely, the near-, mid- and far infrared regions. A chemical species, i.e., an organic molecule, absorbs infrared radiation in the range of 10,000-100 cm\(^{-1}\) and converts it into an energy of molecular vibration. The absorption of the radiation depends on the functional group that the molecule possesses. The absorption of infrared radiation by a functional group occurs at a frequency corresponding to the frequency of the vibration of the functional group itself. Thus, the wavelengths of infrared absorption bands are characteristic of specific types of chemical bonds, and infrared spectroscopy finds its greatest utility for identification of organic and organometallic molecules. Based on the differences in the energy absorbed, the atoms can vibrate in four different ways i.e., stretching, scissoring, bending and rocking. Fourier transform infrared (FTIR) spectroscopy is a measurement technique for collecting infrared spectra. A Fourier Transform Infrared (FTIR) spectrometer obtains infrared spectra by first collecting an interferogram of a sample signal with an interferometer, which measures all of infrared frequencies simultaneously. It, further, acquires and digitizes the interferogram, performs a mathematical fourier transform function, and outputs the spectrum. In this study, we
focused on examining the frequency range from 3000 cm\(^{-1}\) to 3700 cm\(^{-1}\) to verify the formation of multiple hydroxyl groups in SVA [66].

3.2.2.3 Preparation of SVA loaded microspheres

Microspheres loaded with a hydrophilic drug such as SVA were formed using a w/o/w (water-in-oil-in-water) emulsion technique described elsewhere [65]. Briefly, either 25 mg or 50 mg of SVA, according to the theoretical loading of 0.1 and 0.2, were dissolved in 1 ml of distilled-deionized water to constitute the internal aqueous phase. This internal phase was emulsified at 1000 rpm for 45 seconds with 250 mg of PLGA in 2.5 ml of DCM to constitute the initial water-in-oil emulsion. This emulsion was then added to an external aqueous phase (50 ml of 5% poly (vinyl alcohol) (PVA) in 50 ml of distilled-deionized water) with continuous stirring (350 rpm) at room temperature for 10 minutes to form the final w/o/w emulsion. Further, the same protocol, as in the preparation of SV loaded PLGA microspheres, was followed which include solvent evaporation for 5 hours, centrifugation at 2500 rpm, washing with distilled water and freeze drying as described above. The schematic for the double emulsion method is as shown in figure 3.2.
3.2.3 Preparation of the PolyRing Device

The PEG hydrogel was synthesized by chemical crosslinking of poly (ethylene glycol) with triphenyl methane triisocyanate (Desmodur RE) [10]. The procedure involves the addition of 25% (weight) dichloromethane solution of Desmodur RE to 25% (weight) solution of 3350 molecular weight PEG. The molar ratio of the hydroxyl group of the PEG to the isocyanate in the crosslinking agent was varied to obtain the desired crosslinking ratio of 1:1. The solution was then stirred, continuously, at a temperature of 70°C for about 30 minutes to get a fairly viscous solution.

1 g of PLGA microspheres (drug loaded or non drug loaded) suspended in 18 g of DCM had been dispersed in the solution for the PEG hydrogel synthesis after half an hour into its synthesis. The mixture was finally transferred into circular aluminum pans (diameter: 5.8 cm, depth: 0.7 cm) in order to obtain the hydrogel block polymer. The resulting polymer was allowed to stand for a period of 24 hrs at 70°C. Polymeric rings of
approximately 5 mm OD and 3 mm ID were extracted from the block polymer with the use of a drill.

3.2.4 Characterization of the PolyRing device

The drug loaded and non-drug loaded microspheres, prepared by the single and double emulsion techniques, were characterized for surface morphologies, particle size distribution and encapsulation efficiencies. The methods incorporated for each of the above are described in this section.

3.2.4.1 Microsphere Surface Morphology

Scanning electron microscopy (SEM) images of surfaces have great resolution and depth of field, thereby allowing direct imaging of complex microscale structures. In a typical SEM, a high-energy electron beam (typically 5-100keV) is focused on a specimen. When the primary electron emerging from this beam interacts with the sample, low-energy secondary electrons are emitted. The measured intensity of the secondary emission is a function of the atomic composition of the sample and the geometry of the features under observation. These secondary electrons are counted by a detector and is then spatially reconstructed on a phosphor screen (or charge coupled device (CCD) detector) thereby displaying a digital image of the surface. Because of the shallow penetration depth of the low-energy secondary electrons produced by the primary electron beam, only secondary electrons generated near the surface can escape from the bulk and be detected. Therefore, the SEM is primarily used for surface analysis [67].
The surface characteristics of the drug loaded and non drug loaded microspheres were analyzed using a Scanning Electron Microscope (Hitachi & JEOL-JSM35C). A known amount of the microspheres were suspended in distilled-deionized water. 200 µl of this suspension was transferred slowly over an aluminum foil (placed on an aluminum stub using double-sided copper tape) to form a drop covering the entire area of the stub. The stubs were allowed to dry overnight and were further sputter coated with silver and viewed at an accelerated voltage of 15 and 20kV.

3.2.4.2 Microsphere Particle Size Analysis
Laser light scattering experiments were performed to analyze the particle size distribution and to study the effect of drug on the size of the microspheres. This technique measures the size of particles dispersed in a medium by the scattering pattern of a laser light shown through the medium. The size calculations assume the presence of spherical particles [68]. The samples were suspended in a medium consisting of mannitol, carboxymethyl cellulose sodium and polysorbate and the Fraunhofer method was utilized to calculate the size distributions. A known amount of non-drug and drug loaded microspheres were suspended in the above medium and this suspension was subjected to laser light scattering using a laser diffraction particle size analyzer (LSTM™100Q-Beckman & Coulter) at Oakwood Laboratories, LLC.

3.2.4.3 Drug Loading Studies
The loading and encapsulation studies were performed to determine the loading efficiency and the actual loading (formulae listed below) for both the SV and SVA loaded
microspheres. The parameter used to maximize the drug loading was the drug-to-polymer (D/P) ratio. Two D/P ratios, 0.1 and 0.2, were used for both the types of drug loaded microspheres. A known amount of the SV loaded microspheres were dissolved in a solvent blend of ACN:DCM in the ratio of 9:1. Triplicate runs were completed for each type and the drug loading was measured by a spectrophotometric plate reader (Spectramax™) at a wavelength of 225nm. The concentration of the drug was determined from a standard calibration curve for SV in ACN:DCM (9:1).

The SVA loaded microspheres were completely dissolved in DCM and the drug was extracted onto PBS by vortexing and chaotic mixing. Again, triplicate runs were performed and the absorbance of the drug was detected spectrophotometrically at a wavelength of 225nm. The concentration of the drug was obtained from a calibration curve of SVA in PBS. The encapsulation efficiency (expressed as a percentage) was calculated as:

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

Where,

\[
\text{Theoretical drug loading} = \frac{\text{weight of drug}}{\text{initial weight of the polymer}} = D/P \text{ ratio}
\]

\[
\text{Actual drug loading} = \frac{\text{weight of drug (obtained analytically)}}{\text{weight of microspheres}}
\]

3.2.4.4. Microsphere Dispersion (ESEM studies)

An electron microscope requires a good vacuum for the generation and propagation of the electron beam, which implies that the specimen under examination also has to be placed under low pressure conditions. This is a major drawback in conventional SEMs as biological samples and polymers, which undergo significant rearrangement under high
vacuum conditions (e.g., hydrogels), cannot be analyzed. The Environmental SEM (ESEM) allows samples to be observed in low-pressure gaseous environments and high humidity. It utilizes differential pumping systems that allow chamber pressures as high as 2700 Pa and special secondary-electron detectors that operate in the presence of such gas pressures. Fully hydrated samples can be viewed directly as the chamber operates at a pressure equal to the vapor pressure of water at room temperature. The conductive coating of specimens (done for conventional SEMs) is not necessary because the gaseous layer around the specimen becomes ionized and suppresses charge accumulation. The gaseous medium itself acts as a detection medium [67].

The distribution of the drug loaded PLGA microspheres in the PolyRing device was investigated with an Environmental Scanning Electron Microscope (ESEM) (FEI (formerly Philips) Quanta 200) at 30 kV. Swollen PolyRings were loaded onto aluminum stubs. These stubs were examined under low vacuum. The samples were further analyzed for size and location of microspheres in the PEG hydrogel matrix.

3.2.5 Device Sterilization

Gamma sterilization was done at Steris-Isomedix services. The rings were subjected to $^{60}\text{Co}$ gamma-ray irradiation at a dose of 25 kGy (2.5 Mrad).

3.2.6. In vitro drug release studies

Sterilized and un-sterilized PolyRings were transferred to scintillation vials containing PBS (0.01M). The vials were kept in a shaker (Gyratory®, Laboratory Shaker G2) at 100 rpm placed in an incubator (Forma Scientific) maintained at 37°C. Samples were taken
out at defined time points and replaced with fresh buffer. The time points were as follows: 1 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs, 13 hrs, 16 hrs, 20 hrs, 1 day, 2 days, 5 days, 7 days, 10 days, 15 days, 30 days and 45 days. SVA release was monitored by spectrophotometry (Spectramax™) at a wavelength of 225nm. Samples were aliquoted at the above mentioned time points and replaced with equal amounts of fresh buffer to maintain sink conditions. Simultaneously, blank PolyRings i.e., PolyRings with non-drug loaded microspheres were also analyzed at the same time points as mentioned above.

3.2.7 Cell Culture - Smooth Muscle Cell Proliferation Studies

Bovine aortic smooth muscle cells (BAoSMCs), purchased from Cambrex, were subcultured in basal media supplemented with SingleQuots (hEGF, hFGF-B, Insulin, GA-1000 (Gentamicin, Amphotericin-B) and 5% Fetal Bovine Serum) purchased as SmGM-2- Smooth Muscle Medium-2 (BulletKit) from Cambrex. The cells were allowed to proliferate at 37°C in a humidified, 5% CO₂ atmosphere in a Forma Scientific, automatic CO₂ incubator. Cells were passed every week. The cells were used between passages 3 and 6 for the drug and device efficacy studies.

For the drug efficacy comparison study, the cells were plated sparsely in a 12-well flat-bottomed microplate (Costar) at a density of 2000 cells/cm² in the Clonetics smooth muscle cell media system (SmGM-2 BulletKit). Free drug solutions of SV and SVA (at a concentration of 1µM) were introduced to the wells after 24 hours of subconfluency. The media was routinely changed on days 1, 3 and 5. To maintain the drug concentration, free drug solutions, of 1µM concentration, were added each time the media was changed i.e., on days 1, 3 and 5. On days 4 and 7, the cells were trypsinized, according to the protocol
given in Appendix C, and counted using a hemacytometer with trypan blue staining for assessment of cell viability. Trypan blue is a dye that enables easy identification of dead cells. Dead cells take up the dye and appear blue with uneven cell membranes. By contrast, living cells repel the dye and appear colorless. The design for the drug efficacy comparison study is as shown in table I.

Experimental Design:

Control - No treatment

Treatment 1 - Simvastatin drug solution

Treatment 2 - Simvastatin Acid drug solution

<table>
<thead>
<tr>
<th></th>
<th>Day 4</th>
<th>Day 7/90% confluency</th>
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<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Treatment - 1</td>
<td>6</td>
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<tr>
<td>Treatment - 2</td>
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For the device efficacy study, the cells were plated sparsely in a 12-well flat-bottomed microplate with clear inserts (Costar brand transwell-clear inserts with pore size of 3.0 µm) to hold the PolyRing. The cells were plated at a density of 2000 cells/cm² in the Clonetics smooth muscle cell media system (SmGM-2 BulletKit). After 24 hours of subconfluency, the inserts were introduced into the wells with or without the PolyRing according to the design in table II. The gamma-sterilized PolyRings were swollen for 4 hours in sterile distilled-deionized water before being introduced into the wells containing the inserts. The medium were changed routinely on days 1, 3 and 5 with the
cells being trypsinized and counted on the fourth and seventh day using a hemacytometer and the cell viability was determined using trypan blue.

Experimental design:

Control 1: No treatment (Negative control)
Control 2: Simvastatin Acid drug (Positive Control)
Treatment 1: Gamma sterilized PolyRing with no drug.
Treatment 2: Gamma sterilized PolyRing with drug (Simvastatin Acid)
All groups have inserts/filters
Filters/inserts and the devices are introduced after 24 hours

<table>
<thead>
<tr>
<th></th>
<th>Day 4</th>
<th>Day 7 or 90% confluency</th>
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<tbody>
<tr>
<td>Negative Control</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Positive Control</td>
<td>6</td>
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<tr>
<td>Treatment 1</td>
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<td>Treatment 2</td>
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3.2.8. Statistics and Data Analysis

The experiments and studies on microsphere yields, encapsulation efficiencies and actual drug loading amounts were performed in triplicates. The drug loading efficiencies were compared using the Student’s t-test. Values of $p < 0.05$ were considered statistically different. The cell culture studies were performed with six replicates. The data on the plots corresponding to all the experiments conducted were expressed as mean values with the error bars reflecting the standard deviations. To verify the normal distribution of the
data obtained in the cell culture studies, the Shapiro-Wilk test for normality was
performed on each sample group. Each group comprising of six samples was considered
normally distributed when $p \geq 0.05$. Tukey’s analysis of variance was used for multiple
comparisons between the normally distributed groups in the cell culture studies. For
statistically significant difference between the treatments, a $p$ value less than 0.05 was
considered. The statistical analysis were performed on the SAS system (version 9.1), a
computer based statistical analysis system. The results of the statistical analysis are
provided in Appendix A.
CHAPTER IV

DEVICE FABRICATION AND CHARACTERIZATION - RESULTS AND ANALYSIS

The PolyRing system is a composite system which can incorporate anti-proliferative drugs targeted to inhibit IH. This system was designed for local adventitial CyA administration and was successfully developed and characterized by Kanjickal and his co-workers [10, 11]. This work represents the incorporation of the cholesterol lowering drug SV in the PolyRing system for the prevention of IH. Statins have a more direct effect on the inhibition of vascular SMCs in comparison to CyA which acts through the immune system. The two forms of SV used in this study are SV (lactone pro-drug) itself and its active hydroxy acid form SVA which is the potent inhibitor of HMG-CoA reductase [13]. Since the objective is to deliver the drug locally, it is unclear whether the conversion of SV to SVA would occur effectively at the anastomotic site. Therefore, we initially fabricated both SV and SVA loaded PLGA microspheres. Depending on the results of the drug efficacy study (presented in chapter V), which indicated the drug with a comparatively better inhibitory effect, the corresponding drug loaded microspheres were incorporated in the device for further studies. Therefore, the two different microsphere systems (SV and SVA based) were characterized to determine microsphere surface morphologies, size distributions and drug loading efficiencies. Further, the
PolyRing device was characterized for the microsphere distribution. Finally, the in vitro drug release characteristics from this modified PolyRing device and the effects of terminal sterilization on drug release were determined. All the above studies were performed to verify the successful incorporation of statins in the PolyRing system. This chapter describes the results and subsequent analysis of all the different characterization studies performed on the statin incorporated PolyRing system.

4.1. Characterization of SV loaded microspheres

The o/w emulsion technique is based on the agitation of two immiscible liquids i.e., the two phases, organic and aqueous. The drug or the therapeutic agent is either dispersed in the organic phase or is in solution in the polymer/solvent system. Emulsification of the system is continued until the solvent partitions into the aqueous phase and is removed by evaporation. This process results in hardened microspheres which contain the active agent [65]. As mentioned in the methods section in chapter III, the microspheres prepared by this method were characterized for surface morphology, particle size, and drug loading efficiency. This section of the thesis is devoted to the results obtained using the single emulsion method for encapsulating the lactone form of the drug i.e., SV.
4.1.1. Microsphere Yield

According to Jain and others [65], one of the primary requirements for most of the methods of microencapsulation is to obtain a high yield of microspheres. In this work, we determined the yield of microspheres produced using the single emulsion technique. The microsphere yield, expressed as a percentage, was calculated as follows:

\[
\% \text{ Yield} = \frac{\text{Weight of the microspheres}}{(\text{Weight of the polymer} + \text{Weight of Drug})} \times 100
\]

The weight of the microspheres, expressed in mg, was determined post lyophilization. The weights of the polymer and drug, expressed in mg, were the initial amounts of the polymer and drug added in the emulsion process. The parameter used to determine the drug loading is the D/P ratio. Therefore, as mentioned in the methods section in chapter III of this thesis, the microspheres were fabricated using the two D/P ratios, 0.1 and 0.2. Figure 4.1 shows the percentage yield obtained for the SV loaded microspheres.
Fig. 4.1. Percentage microsphere yield for the SV loaded microspheres (n=3)

The % yield obtained for the microspheres with 0.1 and 0.2 D/P ratio was 66% and 85% respectively.

4.1.2. Phase Contrast Microscopy Images

The o/w emulsion based microparticles were analyzed for general morphology using phase contrast light microscopy. A known amount of the microspheres were suspended in distilled-deionized water. This suspension was placed in a small dish and viewed under
the microscope. Figure 4.2 shows the phase contrast micrograph of the blank (non-drug loaded) microspheres fabricated using the o/w emulsion technique.

Most of the particles measured were in the range of 40 to 60 microns. Figures 4.3 and 4.4 depict the SV loaded microspheres fabricated using the two D/P ratios, 0.1 and 0.2 respectively.
A majority of the drug loaded microspheres showed a narrow size distribution in the range of 40-50 microns.
4.1.3 Particle Size Analysis using Dynamic Light Scattering

Among the critical factors that affect drug release kinetics from microspheres, the microsphere size is an important factor. In general, small diameter microspheres result in a faster release due to an increase surface area/volume ratio. Also, the particle size plays an important role in the polymer degradation process. Particles with size <20 microns exhibit diffusion controlled release of compounds such as rhodamine and piroxicam, whereas in larger particles (>50 microns), polymer degradation is the predominant process [69].

DLS is a popular method used to determine particle size. It is based on the principle that when a monochromatic light beam is focused onto a solution containing the particles in Brownian motion, it results in a change in the wavelength of the light. This Doppler shift, that is an outcome of the light striking the particle in motion, is related to the size of particle. The size calculations assume the presence of spherical particles.

Laser light scattering studies were performed to verify the effect of the processing parameters namely, the mechanical stirring speed and the D/P ratio on the size of the microspheres. Particle size tends to increase with the polymer concentration as smaller sized particles are obtained for the lowest phase volume ratio and the highest PVA concentration [46]. With a variable speed impeller, as the speed increases the blades of the impeller induces high shear rates on the droplets thereby resulting in smaller particle size. As mentioned in the methods section in chapter III, the microspheres were suspended in a medium consisting of mannitol, carboxymethyl cellulose sodium and polysorbate and the Frauenhofer method was utilized to calculate the size distributions. Figure 4.5 shows the particle size distribution of blank (non drug loaded) microspheres.
fabricated using o/w emulsion method. The distribution exhibited high polydispersity
with respect to the particle size with the mean of about 100 microns. Almost about (50-75) % of the particles had a particle size in the range of 100 microns (Appendix B). This size distribution encompasses the range of microsphere diameters measured randomly in the phase contrast light microscope image (shown in figure 4.2). The plateau region ranging from 200-400 microns is probably due to the aggregation of the particles in motion.

![Differential Volume (Average)](image)

Fig.4.5. Particle Size Distribution of blank (non-drug loaded) o/w emulsion based microspheres

To analyze the effect of drug loading on the particle size, the drug loaded microspheres were also characterized for size distributions. Figure 4.6 shows the particle size distribution of the SV loaded microspheres produced with a D/P ratio of 0.1.
Fig. 4.6. Particle Size Distribution of SV loaded microspheres with a D/P ratio of 0.1

The mean diameter of these particles was about 45 microns. By comparing the particle size distributions of the drug loaded microspheres with the blank particles, we observe that there is significant decrease in the particle size from a mean of 100 microns to about 45 microns. Therefore, we concluded that the loading of the drug significantly reduced the microsphere particle size. To further investigate the effect of loading the drug on the particle size, we examined the drug loaded microspheres obtained using 0.2 D/P ratio for particle size (shown in Figure 4.7). We noticed that varying D/P ratio did not change the particle size. The microspheres fabricated using 0.2 D/P ratio had a similar particle size distribution as the particles with 0.1 D/P ratio. These particles too had an average particle size of 45 microns. The size distributions, shown in figures 4.6 and 4.7, were similar to the microsphere size measured from the corresponding phase contrast micrographs represented in figures 4.3 and 4.4.
The decrease in the particle size due to the drug loading can be attributed to the decrease in the viscosity of the internal aqueous phase. The addition of the drug to the internal phase i.e. the organic phase probably lowered its viscosity. This could be due to the decrease in the polymer concentration as a results of the drug incorporation in the organic phase \[70\]. This dispersed organic phase would probably be less viscous than the dispersed organic phase with only the polymer and no drug as in the case of blank, non-drug loaded microspheres. This reduction in viscosity would make it easier to be sheared and would also affect the solvent evaporation rate. The rate of the solvent extraction from the emulsion droplets depends on its evaporation from the aqueous phase to air \[71\].

Now, in our study, the parameters such as the stirring speed, phase ratio and the solvent evaporation time were constant for the fabrication of both blank as well as drug loaded microspheres. The viscosity of the organic phase for the production of blank microspheres was probably high which results in a large particle size for the duration of five hours due to a slow solvent evaporation rate. However, as of result of drug
incorporation, the viscosity of the internal phase decreases, and therefore, the emulsion
droplets were continuously reduced in size and this reduction in rapid. Thus, in a span of
five hours, this rapid reduction due to faster solvent evaporation rate resulted in particles
with smaller size. Sansdрап and his co-workers reported the decrease of the particle size
as a function of the organic phase volume. They observed that by increasing the DCM
(organic phase) volume, the diameter of the microspheres decreased. This is due to the
lowering of the organic phase viscosity as the volume of the solvent decreases when the
polymer weight is kept constant [72]. Kim et al., [73], reported a similar observation with
their PLGA microspheres for the sustained release of the recombinant human growth
hormone (rhGH). They observed that by changing the organic/aqueous phase ratio from
3% to 20%, the average particle size decreased from 52.1 microns to 3.8 microns.

4.1.4. SEM analysis for evaluation of microsphere surface morphologies

The o/w emulsion based microspheres, non-drug loaded and SV loaded, were studied for
surface characteristics using Scanning Electron Microscopy. The experimental procedure
related to sample preparation as well as sample analysis has been described in the
methods section of chapter III in this thesis. The main purpose of the SEM analysis is to
study the microsphere surface morphology and the effect of the drug loading on the same.
The SEM micrographs resulting from the study and its interpretation have been provided
in the following paragraphs of this thesis.
4.1.4.1. SEM of non-drug loaded, o/w emulsion based microspheres

Scanning electron micrographs revealed that the o/w emulsion technique yielded spherical particles with a smooth surface structure as shown in figure 4.8. In general, DCM results in particles being spherical and more uniform [74].

![Scanning electron micrograph of blank (non-drug), o/w microspheres (1500x magnification)](image)

Figure 4.8 is a high magnification SEM image of blank (non-drug loaded), o/w emulsion based microspheres. As seen from the image, the surface of the microspheres was smooth and non-porous. Solvent removal rate significantly affects the surface and shape of the microspheres. Removal of the solvent at a fast rate can result in rapid polymer precipitation and result in smoother surface and denser inner structure of the microspheres [75]. The particle size of these blank microspheres was determined using dynamic light scattering described in section 4.1.3 of this thesis.
4.1.4.2. SEM of SV loaded microspheres

The microspheres prepared by o/w emulsion technique were loaded with SV using two D/P ratios, 0.1 and 0.2. Figures 4.9 and 4.10 depict a low and a high magnification scan of the drug loaded microspheres prepared with a D/P ratio of 0.1. Figures 4.11 and 4.12 represent low and high magnification SEM images, respectively, of SV loaded microspheres with a D/P ratio of 0.2.

Fig.4.9. Scanning electron micrograph of SV loaded microspheres with 0.1 D/P ratio (400x magnification)
Fig. 4.10. Scanning electron micrograph of SV loaded microspheres with 0.1 D/P ratio (1000x magnification)

Fig. 4.11. Scanning electron micrograph of SV loaded microspheres with 0.2 D/P ratio (500x magnification)
4.1.5. Encapsulation Efficiency and Actual Drug Loading

One of our primary aims in the development of the microspheres is to achieve sufficient drug loading to have an effect in preventing neointimal formation. This study for analyzing the drug loading efficiencies helped us in verifying the success of our microspheres fabrication techniques. The SV loaded microspheres were fabricated using
a modified o/w emulsion method. This is one of the standard techniques for incorporation of highly hydrophobic drugs [65]. The parameters other than the D/P ratio such as the organic and aqueous phase volumes, polymer weight, stirring speed, emulsification time, and solvent evaporation duration were kept constant. To detect the amount of drug present in the microspheres, a known amount of the drug loaded microspheres were dissolved in a solvent blend of ACN:DCM in a ratio of 9:1. This blend was chosen as the microspheres tend to dissolve much faster in this blend rather than ACN alone. Triplicate runs were performed and the drug was detected using UV-Vis spectroscopy. The amount of drug in each sample was determined by measuring the absorbance spectrophotometrically. The absorbance, obtained as optical density (O.D) values, were recorded and the concentration of the drug was obtained from the calibration curve for SV in ACN:DCM (9:1).

The encapsulation efficiency (expressed as a percentage) was calculated as the ratio of the actual drug loading to the theoretical drug loading. The actual loading was calculated from the detected drug content. The actual drug loading for the microspheres fabricated with the different D/P ratios is shown in Figure 4.13. The drug content in one milligram of microspheres with a D/P ratio of 0.2 was approximately 123 µg. This was statistically higher (P=0.0197) than the drug content present in one milligram of the microspheres with a D/P ratio of 0.1 which was 81 µg.
Figure 4.14 shows the loading efficiencies obtained for the two D/P ratios 0.1 and 0.2. The SV loaded microspheres showed a high encapsulation efficiency of approximately 81%. The microspheres with a theoretical drug loading i.e., D/P ratio of 0.1 resulted in a higher loading efficiency of 81% than the microspheres with a drug loading of 0.2 which resulted in an encapsulation efficiency of approximately 61%. However, there was no statistical significant difference between the loading efficiencies. The higher drug loading might have forced the diffusion of the drug to the aqueous phase which results in a lower encapsulation efficiency.
In general, large microspheres, with a particle size distribution in the range of 80 to 120 microns tend to encapsulate drug more efficiently than medium size microspheres i.e., microspheres in the range of 40-50 microns. The dependence of encapsulation efficiency on particle size is most likely the result of competition between (1) decreased drug diffusion out of the microspheres as the particle size increases or (2) decrease in the rate of drug entrapment due to slower “hardening” of the particles as size increases [69]. Condition 1 tends to an increase in encapsulation efficiencies and condition 2 would lead a decrease in the encapsulation efficiency. In our study, we observed from the particle size analysis in section 4.1.3 that the hardening of the particles was quick which probably
resulted in decreased drug diffusion to the external phase thereby resulting in high loading efficiencies for medium size particles.

A compromise must be found between high encapsulation efficiency and high drug loading [46]. In this work, we decided to proceed with particles with a D/P ratio of 0.1 as this formulation resulted in higher encapsulation efficiency than that obtained with the formulation based on 0.2 D/P ratio. Although, the drug content per milligram of microspheres was higher in latter than the former, due to the restrictions with respect to the availability of the drug, the particles with 0.1 D/P ratio were chosen for further studies on economical basis.

4.2. FTIR analysis for the conversion of SV to SVA
SVA is the pharmacologically active form of SV. This active hydroxy acid form is a potent inhibitor of HMGR [76]. It has been observed that the esterase dependent hydrolysis of SV to SVA in plasma is very rapid in rodents but not in humans and dogs [77]. As the purpose of the PolyRing device is to deliver the drug locally in a controlled manner, it is hypothesized that the active form of the drug i.e., SVA should be incorporated rather than the prodrug (lactone) form i.e., SV.

Among all the statins, only Lovastatin and Simvastatin are inactive lactone prodrugs in oral formulations. These two prodrugs are enzymatically converted to their active hydroxyl acid forms by intracellular enzymes. However, other statins like Pravastatin, Fluvastatin, Atorvastatin, Cerivastatin are administered in their active hydroxy acid form itself. As mentioned in the background of study in chapter II, statins inhibit the mevalonate pathway by the competitive inhibition of the HMGR by binding to
the substrate i.e., HMG-CoA. This competitive inhibition is a result of the HMG-CoA-like moieties (present in all statin molecules) to the corresponding HMG-CoA binding portion of the enzyme active site. However, in all statin molecules (except Lovastatin and Simvastatin), this moiety is an open lactone as shown for Atorvastatin in figure 4.15b [78]. This moiety (highlighted) is similar to that of SVA as shown in figure 4.15a.

![Figure 4.15](image)

Fig.4.15. Structures of (a) Simvastatin Acid (SVA) [79] and (b) Atorvastatin [78]

The moieties in Atorvastatin and SVA are compared to the corresponding moiety in SV (highlighted with dotted lines in figure 4.16) to further explain the structural differences. On comparing figures 4.15 with 4.16, we can clearly observe the difference in the moieties. The lactone ring for SVA and Atorvastatin is open but is in the closed form for SV. To verify the conversion for SV to SVA by FTIR, we focused on analyzing the differences in the spectra corresponding to the structural change in the moieties of SV and SVA.
As mentioned in the experimental section, the IR analysis was performed to verify the conversion of SV to SVA according to the protocol given by Merck. The investigation was carried out using a Nicolet Nexus™ IR spectrophotometer using OMNIC™ software system in order to determine the presence of specific chemical groups in the backbone of the compound. Figure 4.17 shows the FTIR spectrum of SV, the lactone or the inactive form of the drug, considered for this work. The spectrum quite clearly shows the presence of several bands arising from the different interactions of the functional groups.
Fig. 4.17. IR spectrum for SV

The characteristic peak to be observed is the peak arising at a wavelength of 3550 cm\(^{-1}\). This sharp peak depicts the O-H stretching vibrations which are, in general, a characteristic of alcohols and phenols. [66]. This peak indicates the presence of the hydroxyl group in SV, as highlighted in Figure 4.16. The protocol for the conversion of SV to SVA by opening the lactone ring is as described in the methods section of chapter III in this thesis. A large stock solution of SVA prepared according to the MERCK protocol (given in the methods section in chapter III) was used for all the studies pertaining to SVA in this work. The SVA was obtained as a free flowing powder after lyophilization. The powder form of both SV and SVA were used for the FTIR analysis. To verify the conversion, an IR analysis was carried out. SVA is an active hydroxy acid form which is hydrophilic due to the presence of multiple hydroxyl groups resulting from
the opening of the lactone ring as highlighted in Figure 4.15 a. The IR spectrum for SVA is as shown in Figure 4.18.

![Fig. 4.18. IR spectra for SVA](image)

The peak under consideration here is the broad peak covering the range from 3050 cm\(^{-1}\) to 3600 cm\(^{-1}\). This broad peak represents the multiple hydroxyl groups which is characteristic of SVA as shown in figure 4.15(a) as against the presence of a single hydroxyl group for SV (shown in figure 16), which was characterized by the sharp peak at 3550 cm\(^{-1}\) in the IR spectra for SV. The formation of the broad peak for SVA signifies the conversion of SV to SVA. Also, a significant sharp peak present in the IR spectra for SVA and not SV is the peak arising at 1550 cm\(^{-1}\) which represents the carboxylate anion. The carboxylate ion has bond strengths which are intermediate between C=O and C-O. A strong asymmetrical stretching band in the range of 1650 cm\(^{-1}\)-1550 cm\(^{-1}\) is indicative of
the carboxylate ion formation [66]. In SVA, the opening of the lactone might result in the carboxylate ion formation (indicated in figure 4.15 a) as indicated by the sharp peak at 1550 cm\(^{-1}\). Thus, SV was successfully converted to SVA as indicated by the results of the FTIR analysis. SVA was periodically lyophilized from the primary SVA stock solution to be incorporated into the PLGA microspheres.

4.3 Characterization of SVA loaded microspheres

Microencapsulation by the solvent evaporation method is, in principal, quite simple and involves two major steps, the formation of stable droplets of the drug-containing polymer solution and the subsequent removal of solvent from the droplets. In practice, however, the reproducible manufacturing of microspheres with the desired properties (good encapsulation efficiency, suitable release profile and particle distribution, acceptable solvent residuals), can be difficult, due to the large number of factors influencing the outcome, such as solvent composition, total volume and phase volume ratio of the phases, polymer concentration, type of stabilizer, stirring speed, stirring time etc [80].

The double emulsion process (w/o/w) method is best suited to encapsulate hydrophilic drugs, peptides, proteins, and vaccines, unlike the o/w method which is ideal for hydrophobic drugs. These microcapsules are generally smooth and spherical and provide sustained drug release. The characteristics of the microspheres greatly depend on the physiochemical properties of the drug, the polymer and the organic solvent used in the process. The aqueous solubility of the drug is probably the most significant physiochemical property that influences the efficiency of encapsulation in PLGA [65]. In this work, the active form of the drug i.e., SVA was encapsulated in PLGA microspheres.
using a modified double emulsion technique as described in detail in the experimental section. This section of the results focuses on the characterization of the SVA loaded microspheres for percentage yield, surface morphology, and encapsulation efficiency.

4.3.1. Microsphere Yield

The SVA loaded microspheres were fabricated using the double emulsion method by varying the D/P ratio similar to that done for the SV loaded microspheres. The two D/P ratios used were 0.1 and 0.2. Figure 4.19 shows the percentage yield obtained for the SVA loaded microspheres. The percentage yield was calculated according to the equation mentioned in section 4.1.1.

![Graph showing percentage microsphere yield for SVA loaded microspheres (n=3)](image)

Fig.4.19. Percentage microsphere yield for SVA loaded microspheres (n=3)
As shown in Figure 4.19, the percentage yields obtained for the microspheres fabricated using 0.1 and 0.2 D/P ratios were 62±12% and 67±1% respectively.

4.3.2. Phase Contrast Microscopy Images
The w/o/w emulsion based microspheres were analyzed for general morphology using phase contrast light microscopy. A known amount of the microspheres were suspended in distilled-deionized water. This suspension was placed in a small dish and viewed under the microscope. Figure 4.20 shows the phase contrast micrograph of the blank (non-drug loaded) microspheres fabricated using the double emulsion technique.

Fig.4.20. Phase contrast micrograph of double emulsion based blank (non-drug loaded) particles

The presence of two phases is depicted clearly as shown in figure 4.20. Some of the microspheres (lower left in figure 4.20) were composed of multiple reservoirs.
Figures 4.21 and 4.22 represent the phase contrast micrographs for the SVA loaded microspheres with a D/P ratio of 0.1 and 0.2 respectively.

Fig.4.21. Phase contrast micrograph of SVA loaded particles (0.1 D/P ratio)
4.3.3. SEM analysis for evaluation of microsphere surface morphologies

This section of the thesis provides the results and its interpretation obtained from the SEM analysis used to characterize the surface morphologies of the non-drug loaded and drug (SVA) loaded microspheres fabricated using the double emulsion technique. Studying the surface morphologies is important as the surface of a microsphere significantly affects its degradation rate and its interaction with tissues [75]. The experimental procedure related to sample preparation as well as sample analysis has been described in the experimental section of this thesis. As mentioned in the SEM analysis for the SV loaded microspheres in section 4.1.3, the main purpose of the analysis was to study the microparticle morphology and the effect of the drug loading on the same.

Fig.4.22. Phase contrast micrograph of SVA loaded particles (0.2 D/P ratio)
4.3.3.1. SEM of non-drug loaded, w/o/w emulsion based microspheres

Scanning electron micrographs revealed that the w/o/w emulsion technique yielded spherical particles with smooth surfaces morphology as shown in figure 4.23.

As shown in the figure 4.23, two or three visible pores appear on these microspheres. The presence of these few pores is probably due to the local explosion inside the polymer droplets which occurs due to rapid solvent removal [75]. The porosity of the microspheres depends on the rate of precipitation of the polymer and the amount of solvent and water present at the time of precipitation [81].

A rough estimate from the SEM image in figure 4.23 indicates that the microspheres obtained from the double emulsion technique had a large particle size with
diameters in the range of 40-125 microns. The large particle size was probably due to the incorporation of high amounts of PLGA, which itself has a high Mw, thereby resulting in an increased viscosity of the polymer solution. Therefore, the magnitude of shear stress applied during emulsification by the impeller was probably not high enough to break the emulsion into smaller droplets [82, 83]. Thereby, the high viscosity of the organic phase yielded large diameter microspheres.

4.3.3.2. SEM of SVA loaded microspheres

The microspheres prepared by the double emulsion process were loaded with SVA. Figure 4.24 represents a scanning electron micrograph of SVA loaded microspheres fabricated using a theoretical loading (D/P ratio) of 0.1.

Fig.4.24. Scanning electron micrograph of SVA loaded microspheres with a D/P ratio of 0.1 (3600x magnification)
By comparing the scanning electron micrographs in figures 4.23 and 4.24, we observed that the loading of the drug also resulted in microspheres with smooth surface morphologies. However, the spherical geometry of the drug loaded particles was more uniform than the blank (non-drug loaded) microspheres. Furthermore, there were no visible pores on the surface of the SVA loaded microspheres. The SEM image obtained for the SVA loaded microspheres with a D/P ratio of 0.2 is shown in figure 4.25.

![SEM image of SVA loaded microspheres with a D/P ratio of 0.2](image)

**Fig.4.25.** Scanning electron micrograph of SVA loaded microspheres with a D/P ratio of 0.2 (3600x magnification)

The change in the D/P ratio did not seem to affect the morphology of the SVA loaded microspheres. As seen in figures 4.24 and 4.25, the microspheres exhibited smooth surface morphologies and the spherical geometry was maintained. Similar surface morphologies were also observed for somatostatin acetate containing PLGA microspheres reported by Herrmann and Bodmeier. The PLGA used in the above study
had a lactide-to-glycolide ratio of 85:15 similar to the one used for our work [74]. The drug loaded microspheres tend to exhibit a degree of aggregation. The residual amounts of DCM in the microspheres might contribute to the aggregation of these particles during the freeze-drying process [71]. In our study, by visual examination, the lyophilized microspheres seem to be free flowing.

The SVA incorporation significantly affected the particle size of the microspheres. By comparing the scanning electron micrographs in figures 4.23 to 4.25, we observed that particle size reduced from a range of 40-125 microns to about 2-25 microns. The particle size range seen in this work is similar to that obtained by Wei and co-workers. They encapsulated parathyroid hormone in PLGA microparticles and they observed that a surfactant (PVA) concentration of 5% yielded particles in the range of 2-5 microns. The SEM images in their work also revealed a small degree of aggregation similar to our work [84]. A plausible reason for the particle size reduction is explained in the DLS analysis for SV loaded microspheres (section 4.1.3) of this thesis.

4.3.4. Encapsulation Efficiency and Actual Drug Loading
The amount of SVA loaded and the corresponding loading efficiencies were determined in a similar manner to the SV loaded microspheres explained in section 4.1.5. Since, SVA is hydrophilic, the SVA loaded microspheres were initially dissolved in DCM followed by SVA extraction onto PBS by vortexing and chaotic mixing. Figure 4.26 shows the actual drug loading for the microspheres fabricated with different D/P ratios. The drug content in one milligram of microspheres with a D/P ratio of 0.2 was approximately 8 µg.
This was just marginally higher than the drug loading obtained using a D/P ratio of 0.1 which was approximately 7 µg.

Fig.4.26. Actual drug loading for the SVA incorporated microspheres (n=3)

The encapsulation efficiencies of the SVA loaded microspheres are shown in Figure 4.27 shows the loading efficiencies obtained for the two D/P ratios 0.1 and 0.2. We observed rather low loading efficiencies of approximately 7% and 4% for a theoretical loading of 0.1 and 0.2 respectively. The statistical analysis using the student’s t-test revealed no significant difference in the actual drug loading contents and the encapsulation efficiencies. A number of researchers have reported that highly hydrophilic drugs suffer from problems of low affinity with the polymer, leading to an unsatisfactory loading efficiency. If there is poor interaction between the drug and the polymer, the drug
will tend to diffuse from the organic phase to the external aqueous medium during the spontaneous emulsification process of the polymer, thereby leading to poor entrapment efficiencies [85]. In addition to the hydrophilic nature, certain processing parameters may also affect the loading efficiencies. The solvent evaporation time may play an important role in this case. We observed that loading of the drug reduces the particle size of the microspheres. A plausible reason is the decrease in the viscosity which leads to faster precipitation of the polymer during solvent evaporation. This long duration of the solvent evaporation is probably sufficient for the drug to leak out to the external phase thereby leaving very few amounts of drug in the inner phase.

Fig.4.27. Drug loading efficiencies for SVA incorporated microspheres (n=3)
Although, highly water-soluble compounds tend to migrate towards the microsphere surface during the emulsification process, Berkland and others observed that small microspheres kinetically trap the drug resulting in an almost uniform distribution throughout the particle [69]. The efficiency of drug encapsulation in the double emulsion process depends on the rate and the extent of drug diffusion into the external aqueous phase during the solvent evaporation process. The drug diffusion, in turn, is affected by the polymer/solvent interaction and the aqueous solubility of the organic phase [82]. Therefore, the loading efficiency depends primarily on the following factors: (1) primary (w/o) and secondary (w/o/w) emulsion stability, (2) solvent extraction rate, (3) drug, polymer, solvent and surfactant interaction, and (4) particle size [75]. A variation in the above factors can help improve encapsulation of hydrophilic drugs and proteins in microparticles. The study done by Graves and others [68] in blending low and high Mw PLGA to encapsulate the water soluble model drug pentamidine also reveals similar encapsulation efficiencies for the blends containing a higher fraction of the high Mw PLGA. They reported a pentamidine loading efficiency of 9.8% for the microparticles prepared with high Mw PLGA. They observed that the effect of blending high Mw PLGA with low Mw PLGA did not induce an increase in the encapsulation efficiencies to a significant degree except for one formulation which resulted in an encapsulation of about 23%.

The results of the drug encapsulation efficiencies and the actual drug contents revealed in figures 4.26 and 4.27 suggest that increasing the amount of SVA in the emulsion process did not increase the encapsulation efficiency or the drug contents. This is similar to results obtained in the loading of amifostine in PLGA microspheres [82].
The investigators observed that increasing the amount of amifostine in the primary emulsion drastically reduced its loading efficiency. They further incorporated gelatin in the inner aqueous phase to increase the viscosity. They reported that the increase in the viscosity due to gelatin improved the efficiency of amifostine encapsulation thereby proving their theory that increased inner aqueous phase viscosity improves drug encapsulation of hydrophilic drugs as it reduces the drug diffusion during the solvent evaporation process. In accordance to this hypothesis, the reduction in the size of the drug loaded microspheres in comparison to the non-drug loaded microspheres, obtained in our SEM and particle size analysis, is probably due to the decrease in the viscosity of the inner aqueous phase. Therefore, due to the reduced viscosity, the drug rapidly diffused to the external phase resulting in low entrapment efficiencies.

The amount of organic solvent used could have also played a significant role in the low encapsulation efficiency. Low amounts of the organic solvent would result in increased viscosity of the primary emulsion which could slow down the diffusion of the drug to the external aqueous phase. Herrmann and Bodmeier reported increased encapsulation efficiencies with decreasing amounts of organic solvent in the encapsulation of somatostatin in PLGA microspheres by different methods [74]. The low loading efficiency may also be as a result of long solvent evaporation time which allows longer contact and increased drug exchange with the aqueous phase [86]. However, as we wanted to observe the effect of drug in the resulting characteristics of the microspheres, we did not change the processing parameters. The results of the drug encapsulation and loading for SVA loaded microspheres fabricated using the double emulsion process suggest that the increase in the amounts of SVA in the inner aqueous phase decreased the
loading efficiency and did not increase the actual drug content. Therefore, the SVA
loaded microspheres with a theoretical loading of 0.1 were used for further studies in this
work.

4.4. Microsphere Dispersion Studies (ESEM analysis)

The PolyRing is a composite device consisting of the drug loaded PLGA microspheres
embedded in a PEG hydrogel matrix. The procedure for the device fabrication, mentioned
in the experimental section, involved the dispersion of the microsphere suspension in
DCM to the solution for the PEG hydrogel synthesis. The rings were drilled out after 24
hours using drill and plug boring bits. The conventional SEM method cannot be used to
detect microspheres in a swollen hydrogel as the inner chamber in the SEM functions at
high vacuum. A structural change in the device could thus occur due to such unfavorable
conditions. Characterizing the device in the dry state or the un-swollen state is not
favorable either as the microspheres might be embedded too deep in the tight polymer
matrix making it difficult to detect at the surfaces. Therefore, we used the ESEM as it
allows samples to be observed in low-pressure gaseous environments and high humidity.
We investigated the microsphere dispersion in both the dry (un-swollen) device as well as
the fully hydrated (swollen) device using a FEI Quanta 200 ESEM. The unswollen device
was cut axially and the swollen device was manually broken apart along its axial cross-
section. Figures 4.28 represents the environmental scanning electron micrographs of a
single SV loaded microsphere in the PEG matrix. The image reveals that the microsphere
seemed to be partially embedded in the matrix making its complete visualization difficult.
Figure 4.29 to 4.30 depict the environmental scanning electron micrographs of the fully hydrated cross sections of the PolyRing in increasing magnifications.

Fig.4.28. Environmental scanning electron micrograph of the unswollen matrix at 1515x magnification
Fig. 4.29. Environmental scanning electron micrograph of swollen axial cross section of the device at 373x magnification
Fig. 4.30. Environmental scanning electron micrograph of swollen axial cross section of the device at 868x magnification.
The ESEM images for the hydrated devices revealed a uniform distribution of the microspheres throughout the hydrogel matrix as observed across the axial cross-sections shown in figures 4.29. A careful examination of figures 4.29 suggests the presence of a few craters that correspond to location of the microspheres. The microsphere size, measured in the image itself with its appropriate scale bar (figure 4.30), was in the range of approximately 30-50 microns. These size measurements were consistent with our particle size distributions obtained for SV loaded microspheres described in section 4.1.4 of this chapter. Therefore, the PolyRing device synthesis process does not seem to affect the size and morphologies of the drug loaded microspheres. To our knowledge, this is the first time any perivascular based polymeric drug delivery system has been characterized for microsphere distribution within the hydrogel matrix. Thus, the device has been successfully characterized using ESEM.

4.5 In vitro drug release studies

The in vitro drug release studies were carried out to determine the length of drug delivery and to elucidate the release characteristics. Almost all the previous research concerning SV and SVA, state that SVA, the active hydroxy acid form of SV, is the potent competitive inhibitor of the HMG-CoA reductase [13, 76, 77, 79]. In accordance with the above mentioned literature, we decided to examine the in vitro release profiles of SVA. This section depicts the SVA release profiles from un-sterilized and gamma-sterlized PolyRings obtained from the studies performed at 37°C, the physiological temperature.
4.5.1 Release characteristics of SVA from unsterlized PolyRings

The solute (SVA) transport studies from both the unsterilized and gamma-sterilized PolyRings were studied. The release medium or the buffer used to study the drug release profiles was PBS. PolyRings immersed in PBS were maintained at 37°C to simulate body conditions. Figure 4.31 depicts the dissolution profile of SVA from unsterilized PolyRings. A triphasic release profile was observed over a period of 70 days. The release was performed in triplicates with concentrations determined twice for each sample. Therefore, each data point on the release plot was an average of six measurements. The initial rapid release of the drug occurred over the first 24 hours in the release medium. This represents the burst release with more than 30% of the drug releasing in this time period. This phase is due to the dissolution of the drug present on the surface of the microspheres which in turn is due to the rapid diffusion of the aqueous buffer into the polymeric matrix [83]. The second phase, which is almost linear, corresponded to the lag period of slow release over an extended period of time. This slow release of the drug depends on the degradation of the microspheres and the diffusion of drug, present in the inner core of the microspheres (encapsulated drug), through the polymer matrix. The complete erosion of the polymeric matrix represented the third and final phase of the release which occurs over 50-70 days. The erosion results in an increase in the release rate.
Fig. 4.31. Percentage release of SVA from unsterilized PolyRings. Each point is an average of six measurements from three replicates.
The initial release or the phase corresponding to the burst effect is defined as the amount of drug released over a span of 24 hours. This phase plays an important role in determining the therapeutic efficacy of the system [87]. In the release profile of SVA from unsterilized PolyRings, the burst phase resulted in approximately 30% of the drug releasing over 24 hours. This is comparatively lower to the percentage of drug released in the initial phase observed by other investigators. For instance, Zilberman and Shraga reported a 40-50% of horseradish peroxidase (HRP) release from fiber/microsphere composites consisting of PLGA microspheres (inherent (inh) viscosity of 0.65 dl/g) in the burst release of 24 hours [83]. Graves et al.,[68] investigated the effects of high and low Mw PLGA blends on the release characteristics of pentamidine encapsulated microcapsules. The formulation with a high Mw PLGA (inh viscosity of 0.8 dl/g) results in more than 50% of pentamidine release within 6 days. This percentage of drug release is marginally higher than our results over the same time period. Although the SVA loaded microspheres had a drug loading efficiency of only around 7%, we achieved a sustained release of the drug over almost 50-70 days with only 30% of drug being released in the first 24 hours. This restricted diffusion of the drug can be attributed to the PEG hydrogel matrix. The hydrogel matrix probably acts as a barrier by preventing the initial burst release of the drug to a certain degree.

Certain factors like the theoretical loading significantly affects the initial drug release rates as observed by Pamujula et al [82]. They reported an increase in the percentage of amifostine release from PLGA microspheres with an increase in the amount of drug incorporated in the inner aqueous phase. To increase the theoretical loading, higher amounts of drug needs to be incorporated in the inner aqueous phase.
This increase in the drug concentration increases the concentration gradient thereby forcing the drug diffusion to the external phase. This enhanced diffusion contributes to the high surface localization of the drug, which in turn increases the initial drug release. A prolonged release of the therapeutic agent is desirable for preventing IH, as it increases the effectiveness of the inhibition [53, 88].

4.5.2 Release Characteristics of SVA from gamma-sterilized PolyRings

From a biological point of view, terminal sterilization is preferred over aseptic processing. Aseptic processing in clean room environments is costly and labour intensive. In addition to these factors, it is more vulnerable with respect to microbiological contamination of the finished product [89]. In general, PLGA undergoes radiolytic degradation during gamma irradiation and the Mw of PLGA tends to decrease resulting in a higher release rate [90]. The size exclusion chromatographic results from the in vitro microsphere degradation studies in the preceding work with CyA indicated that the molecular weights of the PLGA microspheres embedded in the sterilized and the unsterilized rings were not significantly affected by the sterilization process [10].

Kanjickal et al.,[10] reported that the complexity of the drug molecule plays an important role in understanding the effects of sterilization. They observed that the CyA release from PEG hydrogels was significantly affected by sterilization. However, they reported that the release of rhodamine from PEG hydrogels wasn’t affected by sterilization. CyA is hydrophobic with a Mw of 1202.6 Da whereas Rhodamine is hydrophilic with a Mw of 479 Da [10]. Now, SVA is also hydrophilic with a MW of 436 Da [79]. Therefore, we expected that the release profiles of SVA would not be altered by
gamma-sterilization. To verify this, we performed the drug release studies of SVA from gamma-sterilized PolyRings at 37°C in PBS. Figure 4.32 depicts the release of SVA from gamma-sterilized PolyRings. The release was performed in triplicates with concentrations determined twice for each sample. Therefore, each data point on the release plot was an average of six measurements.
Fig. 4.32. Percentage release of SVA from gamma-sterilized PolyRings. Each point is an average of six measurements from three replicates.
The release profile for the sterilized PolyRings (shown in figure 4.32) seems to follow a similar trend as observed with SVA release from unsterilized PolyRings (Figure 4.31). Gamma sterilization resulted in a marginal increase in the initial burst release rate, but did not affect the release rates in the subsequent phases. Such an effect was also observed by Faisant et al [91] in their investigation corresponding to the release of Fluorouracil loaded PLGA microparticles. Comparing the release profiles obtained for unsterilized and gamma-sterilized PolyRings in figures 4.32 and 4.31, we conclude that gamma-sterilization did not affect the release of SVA from the PolyRing system.
CHAPTER V

SMOOTH MUSCLE CELL PROLIFERATION STUDIES - RESULTS AND ANALYSIS

Inhibition of vascular SMC proliferation is one of the primary in vitro studies for determining the efficiency of a drug delivery system incorporated with anti-proliferative drugs for preventing conditions like restenosis and IH. In this work with SV, we hypothesized the need to incorporate the active hydroxy acid form i.e. SVA as against the lactone pro-drug SV. The oral formulation consists of the lactone form which converts to the active hydroxy acid form for pharmacological action. Since, we are focusing on the local inhibition of vascular SMCs, we performed a drug efficacy cell culture study to compare the inhibitory effects of SV and SVA. This chapter describes the results obtained from two different cell culture studies i.e., the drug comparison study and the device efficacy study. In the first section of this chapter, the results of the drug efficiency study performed to determine and compare the cell proliferation inhibitory effects of SV and SVA on SMCs are presented. The drug with the higher inhibitory effect was incorporated in the PolyRing device and the second phase of the cell culture studies was performed to determine the efficacy of the device in inhibiting cell proliferation in culture. The results of the second phase of the cell culture studies are described and discussed in the second and last section of this chapter.
5.1. Cell culture studies for comparing the efficacy of the drugs in inhibiting smooth muscle cell proliferation

The HMG-CoA reductase inhibitors are known to be the most effective class of drugs to reduce serum cholesterol levels because of their ability to competitively inhibit the action of the HMG-CoA reductase enzyme which is the rate limiting enzyme in the synthesis of cholesterol. This reduction in the cholesterol levels has reduced the risk of cardiovascular events [12]. As a result of the inhibition of the HMG-CoA reductase enzyme, statins also prevent the catabolism of the isoprenoid intermediates of the mevalonate pathway i.e., FPP and GGPP. These isoprenoids are vital for multiple cellular functions as they are important lipid attachments for the post-transitional modification of a variety of proteins, including Ras and Rho GTP-binding proteins. The Rho protein plays an important role in the cytoskeleton organization, motility, secretion, proliferation and cell signaling [12]. Therefore, the prevention of the isoprenoids results in a number of cell based pleiotropic effects of statins.

The migration and proliferation of vascular smooth muscle cells are primarily responsible for neointimal thickening. Independent of their lipid lowering properties, statins have shown to reduce the migration and proliferation of smooth muscle cell in vitro. The exact mechanism of the inhibition of vascular smooth cell proliferation by statins is described in detail in the chapter II comprising the background section of this thesis. All statins, with the exception of Lovastatin and Simvastatin, are administered as the active β-hydroxy acid form. Lovastatin and Simvastatin are administered orally as lactone prodrugs which are converted to the corresponding β-hydroxy acid forms by the action of enzymes in vivo. The hydroxy acid forms of both these drugs are
pharmacologically active [78]. The structures of the β-hydroxy acid of other statins and the prodrug SV are compared in the results of the FTIR analysis in section 4.2 of chapter IV in this thesis. According to the researchers at Merck [13, 76], SVA, the active hydroxyl acid form of the prodrug SV is the potent inhibitor of the HMG-CoA reductase enzyme. The PolyRing device incorporated with Simvastatin is applied at the site of the anastomosis, i.e. at the site of IH, to locally deliver the drug in a sustained manner. Therefore, since we are applying the device locally, we hypothesize the need of delivering the active form (SVA) as against the lactone form (SV).

Statins exhibit a number of pleiotropic effects described in chapter II comprising the background section. Our aim is to utilize the efficiency of statins in inhibiting the smooth muscle cell proliferation. Our hypothesis was that the efficacy of SVA in inhibiting the smooth muscle cell proliferation is significantly higher than the efficacy of SV in the inhibition of the same. To test our hypothesis, we performed a cell culture study to compare the efficacy of SV versus SVA in reducing cell proliferation. The cells incorporated for this study were the bovine aortic smooth muscle cells (BAoSMCs) used between passages 3 and 6. The cells were plated at a density of 2000 cells/cm$^2$ in a 12-well flat-bottom plate. The design for the experiment is as shown in Table I in the methods section of chapter III in this thesis. Twelve wells (six each for day 4 and day 7) had no treatment (control), twelve wells had been treated with SV (treatment 1) at a concentration of 1 µM, and twelve wells were treated with SVA (treatment 2) at a concentration of 1 µM. The cells from all the three groups were harvested on days 4 and 7 for counting. Figures 5.1 and 5.2 shows the cell counts obtained for the control and treatments on Day 4 and Day 7 respectively.
Fig. 5.1. Cell counts obtained on day 4 - drug efficacy comparison study (n=6)
Fig. 5.2. Cell counts obtained on day 7 - drug efficacy comparison study (n=6)
The results of the cell counts obtained on day 4 indicated significant differences between all the three groups. The live cell count data in each of the three groups i.e., control, treatment 1 (SV), and treatment 2 (SVA) were tested for normality using the Shapiro-Wilk test. All the three groups were normally distributed with the P values greater than 0.05. The sample programs and the corresponding statistical results are shown in Appendix A. To compare the means, the Tukey’s analysis of variance was used. The computations were performed using the SAS (Version 9.1) software. The Tukey’s comparison revealed statistical significant difference between the three groups. The number of live cells/ml was significantly higher in the control group than that in the treatments 1 and 2 with P values of 0.0122 and <0.0001. The number of live cells/ml in the group with SVA treatment was significantly lower (P<0.0001) than the number of live cells/ml in the SV treated group. This implied that SVA was more efficacious than SV.

As seen in figure 5.2, the data obtained on day 7 further corroborates the data obtained on day 4. The data within each of the three groups were normally distributed as tested by the Shapiro-Wilk test for normality. The Tukey’s test showed significant difference between the three groups. The cell count obtained for the control group was significantly higher than the groups with SV and SVA treatments with P values of 0.0004 and <0.0001 respectively. As seen with the results for day 4, the inhibitory effect of SVA was again significantly higher than SV (P<0.0001) on day 7. Therefore, the active form (SVA) is more efficacious in preventing smooth muscle cell proliferation than the lactone prodrug SV. However, SV too inhibits the cell proliferation in comparison to the control groups which suggest that SV too affects the cell proliferation, but, to a lesser degree than
SVA. This effect could be attributed to the probable enzymatic conversion of SV to SVA by the action of the cells in the culture environment.

Figures 5.3, 5.4, and 5.5 depict the cell morphologies on day 7 of the control, SV treated, and SVA treated groups respectively. The images were taken using a digital camera (Nikon D80) by observing the wells under an optical microscope (Zeiss). The figures depict no structural differences between the SMCs in the controls and the drug treatments indicating lack of SV or SVA possible toxicity.

Fig.5.3. Optical Image of cell morphology on Day 7 - Control Group (16x magnification)
Fig. 5.4. Optical Image of cell morphology on Day 7 - Trt 1 (SV) (16x magnification)

Fig. 5.5. Optical Image of cell morphology on Day 7 - Trt 2 (SVA) (16x magnification)
According to Laufs and others, there are no observable adverse affects of Simvastatin under a dose of 10\(\mu\)M on cell viability. Levels of more than 20\(\mu\)M were reported toxic [34]. The dose of 1\(\mu\)M was used as it was within the limits of being non-toxic. In addition, the solubility of SV in water is very low. The solutions of the two drugs were prepared in sterile distilled-deionized water. Therefore, the maximum concentration of SV that could be achieved in aqueous solutions was about 1 \(\mu\)M. Therefore, the concentration of the SVA solution in sterile distilled-deionized water was also maintained at 1 \(\mu\)M.

In most of the research work pertaining to studying the effects of statins on SMC proliferation, SV has been converted to be used in the active form i.e., SVA. Laufs et al.[34] activated SV to SVA to determine the contribution of isoprenoid synthesis to the PDGF-stimulated SMC cycle progression by treating the cells with increasing doses of SVA in the range of 1 to 10\(\mu\)M. SV was converted to SVA by treating with NaOH and HCl and used in the cell culture of rat aortic SMCs (RAoSMCs) to study the effect of SV on SMC proliferation in vitro and neointimal formation in vivo after injury to the vasculature. The investigators observed that a local delivery of mevalonate prevented the inhibitory effects of SV in vivo by increasing the neointima-media ratio [38]. Wiesbauer and co-workers observed a cytotoxic effect of SVA at a concentration of 5\(\mu\)M on human umbilical vein endothelial cells. However, at concentrations lower than 5\(\mu\)M, the cell viability wasn’t affected [92]. Corpataux et al.,[93] performed a study to investigate whether the inhibitory effects of all the statins on SMC proliferation is a class effect or only confined to specific statins. SV was used at a concentration of 1\(\mu\)M, and activated to SVA. All the statins seemed to significantly reduce the human saphenous SMC
proliferation, migration, and invasion with Fluvastatin exhibiting the highest inhibitory effect. Sindermann and others [94] studied the effects of Lovastatin and SV on the proliferation and viability of RAoSMCs and BAoSMCs. The active forms of SV and Lovastatin were used at concentrations of 1-20µM. They observed that the cell proliferation, studied by cell counting and [3H] thymidine incorporation was significantly inhibited by Lovastatin (active form) and SVA with the effect of SVA being 2-3 folds stronger than Lovastatin.

The inhibitory effect of SVA was much higher than SV as indicated by the drug efficacy comparison studies. Therefore, the PolyRing device incorporated with SVA loaded microspheres were used for the further studies to determine the efficacy of the SVA incorporated device in the inhibition of SMC proliferation in culture. Furthermore, the outcome of this study justified our decision of studying the in vitro release characteristics of SVA.

5.2 Smooth Muscle Cell Proliferation Studies to test the efficacy of PolyRing incorporated with SVA

The process of neointima formation is primarily due to the migration of smooth muscle cells from the media to the intima. Fibroblasts from the adventitia also tend to migrate to the media and intima and aid intimal thickening. Therefore, the adventitial delivery of therapeutic agents seems justifiable [5]. Furthermore, high systemic concentrations of therapeutic agents may also lead to potential side effects. We have, therefore, developed a perivascular polymeric drug delivery device incorporated with SV, a cholesterol lowering drug, to inhibit IH locally. The drug efficacy study performed to compare the inhibitory
effects of SV and SVA revealed that SVA was more efficient than SV in preventing SMC proliferation. Therefore, the device incorporated with SVA was tested for its efficacy in inhibiting SMC proliferation. For the successful inhibition of IH, in 1990, Edelman and others [53] had suggested that controlled heparin release from polymeric matrices placed on the injured arterial segments adventitiously could be efficacious in preventing the proliferation of vascular SMCs following endothelial injury. The rationale behind delivering heparin for a prolonged period of time was to reduce the risks associated with systemic therapy since the doses used for systemic administration could result in hemorrhage and thrombocytopenia. They investigated the effects of traditional intravenous heparin administration versus the effects of controlled adventitial heparin release. They reported data in terms of the percentage inhibition of luminal occlusion. The heparin released peri-adventitially resulted in 85% inhibition of occlusion. In contrast, the intravenous administration resulted in only 70% inhibition of occlusion and the inhibition by sub-cutaneous heparin administration was even lower at about 50%. Therefore perivascular heparin administration was more efficacious than the intravenous and the sub-cutaneous routes.

In the device efficacy study, all the four groups (two treatments and two controls) had microporous inserts used to keep the device in place. The design for the SMC proliferation study to test the efficacy of the SVA loaded PolyRings is described in Table II in the methods section of chapter III in this thesis. As described, twelve wells had drug (SVA) loaded PolyRings, twelve wells had non-drug loaded PolyRings incorporated with blank PLGA microspheres. As mentioned in the methods section, the PolyRings were swollen for four hours in sterile distilled-deionized water before being introduced along
with the inserts into the wells. The concentration of SVA in each PolyRing was estimated to be approximately 38µg. Both these treatments had the devices being held by the microporous inserts. Twelve positive control wells had inserts with the free drug (SVA) at a concentration of 1 µM. Twelve wells had only the inserts representing the negative control. The cells were harvested on days 4 and 7 for counting. Figures 5.6 and 5.7 show the results pertaining to the cell counts obtained on days 4 and 7.
Fig. 5.6. Cell counts obtained on day 4 - device efficacy study (n=6)
Fig. 5.7. Cell counts obtained on day 7 - device efficacy study (n=6)
As observed from the results of the cell count obtained on day 4 (shown in figure 5.6), the four groups (two treatments and two controls) were significantly different from each other. The live cell count data in each of the four groups i.e., negative control, treatment 1 (Blank PolyRings), treatment 2 (SVA loaded PolyRings), and positive control were tested for normality using the Shapiro-Wilk test. All the four groups were normally distributed with the P values greater than 0.05. The sample program and the corresponding statistical results are shown in Appendix A. The Tukey’s analysis revealed that there was a significant difference between the two treatments (P=0.0003) indicating the inhibitory effect of the drug loaded device. The live cell count in the negative control was significantly higher than the drug loaded as well as the non-drug loaded devices (P<0.0001 and P=0.0001 respectively). The results from day 7 are shown in figure 5.7. The four groups were normally distributed as tested by the Shapiro-Wilk normality analysis. Similar to the results obtained on day 4, the live cell count obtained for treatment 2 (drug loaded device) was significantly lower than the treatment 2 (non-drug loaded device) (P<0.0001) and the negative control (P<0.0001). The effect of the drug loaded device seemed to increase from day 4 to day 7. This probably indicated the effect due to the sustained release of the drug. The cell count obtained for the drug loaded devices on days 4 and 7 were statistically higher than the positive control (free drug) indicating the dosage is less than 1 µM. The cell viability for both the treatments were high as seen in figures 6 and 7 (live and dead cell count) indicating no long-term effect/toxicity of the drug as well as the device.

An interesting observation is that there was a significant difference between the treatment 1 (non-drug loaded device) and the negative control on day 4 (P=0.0001).
However, there was no significant difference between the former and latter on day 7 (P=0.8434). This was probably due to the dilution effect of the device on day 4 which results in the difference in the cell counts. The PolyRings were swollen in sterile-distilled deionized water prior to being placed in the inserts. Due to the concentration differences between the swollen PolyRing and the surrounding media, an active exchange of water (from the PolyRing) and cell media occurred resulting in a dilution of the media. This dilution could have reduced the nutrients available to the cells thereby causing them to proliferate slowly. As the media is replenished at regular intervals, this dilution effect was diminished over time. This result further corroborates the cytotoxicity study done by Kanjickal et al. [10] indicating the non-cytotoxicity of PolyRing device incorporated with statins.

In the tissue culture experiments performed by Edelman and others to test the efficacy of non-anticoagulant heparin and anticoagulant heparin, the effects of both the drugs in the inhibition of SMC proliferation was equal but the non-anticoagulant heparin achieved a therapeutic response only when delivered adventitially [95]. Edelman and his co-workers further continued their work on perivascular systems by incorporating heparin loaded PLGA microspheres in alginate films. The results from their cell proliferation studies revealed that the percentage inhibition with respect to alginate films containing no heparin increased in a dose dependant manner [60]. Fishbein et al.[58], developed EVA matrices incorporated with tryphostin AG-1295 to assess the antirestenotic effect of the therapeutic agent in a rat carotid model by a site-specific sustained release system. They observed significant reduction in rat aortic SMC proliferation due to the effect of AG-
1295 at concentrations of 5, 10 and 50 µM. This dosage range is much higher than the dose of SVA used in our study.

Figures 5.8 to 5.15 depict the cell morphologies obtained for all the four groups on days 4 and 7 respectively. Comparison of the cell morphologies between the control and the treatments revealed no alterations in the cell structure or growth pattern further indicating lack of device induced toxicity.
Fig. 5.8. Cell morphology for the negative control - Day 4 (16x magnification)

Fig. 5.9. Cell morphology for the positive control - Day 4 (16x magnification)
Fig. 5.10. Cell morphology for treatment 1 (non-drug loaded device) - Day 4 (16x magnification)

Fig. 5.11. Cell morphology for treatment 2 (SVA loaded device) - Day 4 (16x magnification)
Fig. 5.12. Cell morphology for negative control - Day 7 (16x magnification)

Fig. 5.13. Cell morphology for positive control - Day 7 (16x magnification)
Fig. 5.14. Cell morphology for treatment 1 (non-drug loaded device) - Day 7 (16x magnification)

Fig. 5.15. Cell morphology for treatment 2 (SVA loaded device) - Day 7 (16x magnification)
CHAPTER VI
CONCLUSIONS AND FUTURE WORK

6.1 Conclusions
The high failure rates for hemodialysis access are primarily due to the development of IH at the anastomotic sites. IH is characterized by the thickening of the intima due to the migration and proliferation of smooth muscle cells from the media. Localized adventitial delivery of antiproliferative drugs have shown to be efficacious in preventing intimal thickening [95]. Kanjickal and others [10, 11] developed a perivascular polymeric controlled drug delivery system to address the issue of IH. This system, named PolyRing, incorporated with the immunosuppressant CyA. Since the action of CyA is dependant on the immune system, we modified the PolyRing system by incorporating statins in it. Statins, a group of cholesterol lowering drugs, were chosen due to their direct inhibitory action on smooth muscle cell proliferation. This work focuses on the characterization and in vitro analysis of the PolyRing system incorporated with Simvastatin (SV) (ZOCOR®).

The research hypothesis stated that the lactone pro-drug (SV) or the active hydroxy acid (SVA) can be incorporated into the PolyRing device for sustained drug delivery applications. To test this hypothesis, we characterized the drug incorporated device for microsphere surface morphologies, particle size distribution and determined the drug loading efficiencies. The device, itself, was visualized by ESEM to determine
whether the microspheres were dispersed in the PEG hydrogel matrix. Further, in vitro release studies at 37°C, the physiological temperature, were performed to determine the duration of drug release. The results of the microsphere and device characterization studies indicated that both the drug forms (SV and SVA) had been successfully incorporated in the PolyRing system. The SV loaded PLGA microspheres, prepared by the o/w emulsion technique, had spherical geometries with smooth surfaces morphologies as analyzed from the scanning electron micrographs. These microparticles had a size distribution in the range of 40-60 microns. A high loading efficiency of 81% was obtained for the microspheres fabricated using the single emulsion technique. A double emulsion technique was used to encapsulate the hydrophilic acid form of SV i.e., SVA in PLGA microspheres. The w/o/w method of fabrication resulted in small diameter (2-25 microns) SVA loaded microparticles exhibiting spherical geometries with smooth surfaces. However, a rather low encapsulation efficiency of approximately 7% was obtained for the SVA loaded microparticles owing to partitioning of the drug to the external aqueous phase. The encapsulation efficiencies of highly water soluble drugs are often low due to the lower affinity of the drug to the polymer and a greater affinity towards the external aqueous phases which forces its diffusion outwards leading to poor drug loading [68, 85]. Furthermore, the method of determining the drug loading amount isn’t always accurate due to factors like aggregation at the interface of the aqueous and organic phases during extraction, and incomplete recovery [83]. Drug loading affected the particle size by reducing the diameter of the microparticles. The incorporated drug probably decreases the inner phase viscosity which resulted in particle size reduction. This effect was observed both for SV as well as SVA loaded microspheres. Both the
emulsion techniques resulted in a high microsphere yield in the range of 60-85%. The PolyRing device characterized by ESEM revealed the distribution of the microspheres throughout the PEG hydrogel matrix. To our knowledge, this is the first study to characterize microsphere dispersion in the hydrogel matrix of a perivascular polymeric controlled drug delivery system. The in vitro drug release studies indicated that a sustained release of SVA was achieved from the PolyRing system comprising of a characteristic burst release followed by prolonged drug diffusion and polymer degradation phase over a long duration. The release lasted for about 70 days. The release profiles for sterilized PolyRings suggested that SVA release was minimally affected by gamma sterilization. Therefore, the above mentioned results indicated that both drug forms (SV and SVA) have been successfully incorporated in the PolyRing system for sustained drug delivery applications.

According to Duggan and others [13], SV is a lactone prodrug which is enzymatically converted to its active form i.e., SVA in vivo. This active hydroxy acid form is the potent inhibitor of the cholesterol pathway. Since, we are delivering the drug locally, we hypothesized the need of incorporating the active form (SVA) rather than the lactone form (SV). Our null hypothesis 1 stated that there is no significant difference in the inhibitory effects of SV and SVA in preventing the proliferation of vascular smooth muscle cells in culture i.e., there is no significant difference between the live cell counts for samples treated with SV and SVA. An in vitro cell culture study was used test this hypothesis. The study was performed on bovine aortic smooth muscle cells (BAoSMCs). The experimental design consisted of three treatment groups, i.e. one group with no treatment, one with free SV, and one with free SVA. The cells were counted on days 4
and 7 to determine the inhibitory effects. The statistical analysis on the live cell counts, on both time points i.e., day 4 and day 7, indicated a significantly higher inhibitory effect of SVA than SV. Therefore, these results indicated the superior inhibitory effect of the active form (SVA) than the lactone form (SV). Furthermore, the outcome of this drug efficacy comparison study confirmed our justification of studying the in vitro release profiles of SVA rather than SV. Thus, we fail to accept our null hypothesis 1 and thereby reject it.

The PolyRing system incorporated with SVA was tested for its efficacy in the prevention of smooth muscle cell proliferation. The null hypothesis 2 stated that the drug loaded PolyRing system does not have a significant effect in preventing vascular smooth muscle cell proliferation in culture i.e., there is no significant difference between the live cell counts for samples treated with the drug-loaded PolyRings and non-drug loaded PolyRings. In accordance with the results obtained in the drug efficacy comparison study, we incorporated SVA loaded microspheres in the PolyRing system. This device efficacy study, performed on BAoSMCs, was designed to have two treatments and two controls. The positive and negative control groups received free drug (SVA) and no drug respectively. The two treatments, treatments 1 and 2, were incorporated with the blank microspheres loaded PolyRings (non-drug loaded PolyRings) and SVA loaded PolyRings respectively. The cells were counted on two different time points, day 4 and day 7. The results of the statistical analysis pertaining to the device efficacy study revealed that the SVA loaded PolyRing device significantly inhibited smooth muscle cell proliferation in comparison to the non-drug loaded PolyRings and the negative controls. However, there was no significant difference in the live cell counts obtained for the non-drug loaded
PolyRings and the negative controls on day 7 confirming that the PolyRing system incorporated with SVA is non-cytotoxic. This result indicated that a significant inhibitory effect was achieved although the drug loading efficiencies were reportedly low. Therefore, SVA incorporated PolyRing system successfully inhibited the proliferation of SMCs in culture. Thus, we fail to accept our null hypothesis 2 and therefore reject it.

Statins are also known to induce vascular SMC apoptosis. However, according to Erl et al.,[39, 96] this apoptotic effect of statins is more preferential towards the SMCs of the neointimal origin than the medial origin. In their in vitro culture studies to mimic early stages of neointimal formation, they observed that the treatment with statins led to neointimal cell death but not medial SMC apoptosis. Therefore, the apoptotic effect of statins is primarily confined to the neointimal cells. In addition to this finding, Guijarro et al., [37] compared the effects of different statins on rat vascular SMC apoptosis. They reported that SV induced significant levels of apoptosis only above 10 μM. In our cell culture studies, we used the commercially available BAoSMCs and the positive control used was SVA at a concentration of 1 μM. Therefore, we did not observe any cell death due to the origin of the cell line and the concentration of the drug used which was well below its level to induce apoptosis.

The results of the characterization studies indicated the successful incorporation of SV and SVA in the PolyRing system. The in vitro studies of the SVA loaded PolyRings have achieved a significant degree of success in this work as observed with the results corresponding to the in vitro drug release and cell culture studies. Therefore, the PolyRing system developed by Kanjickal and others has been successfully modified by incorporating the cholesterol lowering SV in it. The outcome of this study indicates that a
variety of other drugs or therapeutic agents could be incorporated in the PolyRing system for sustained release applications. The release of SVA from the PolyRing system lasted for about 70 days. In accordance with the standardized balloon injury model by Clowes and others, the extent of IH is maximal, reaching a plateau at 14 days after the procedure [18, 19, 38]. Therefore, in most of the animal studies pertaining to studying the effects of antiproliferative drugs on IH, the analysis is done at 2-3 weeks after the procedure or graft implantation [49, 57-60, 62]. Since the intimal SMC proliferation in animal models of vascular injury is maximal in extent at 10-14 days following injury, we suggest that the PolyRing system incorporated with statins could be effective in preventing the SMC proliferation as 63% of SVA releases from the PolyRing system within the first 15 days.

6.2 Future work

The SVA loading efficiency in our work was observed to low. In order to improve its loading efficiency, certain fabrication parameters such as the volume of the organic solvent and the viscosity of the polymer solution could be varied to observe its effects on the encapsulation efficiencies [68, 74]. Along with the above parameters, the effects of varying the pH or the internal and external aqueous phases could also play a significant role in improving encapsulation efficiencies [81].

The next phase of work is to evaluate the in vivo safety and biocompatibility of the statin loaded PolyRing system. This could be achieved by performing animal studies on the PolyRing. The device could be placed on arterial and venous sites and could be monitored over a period of time. The tissue sections from the implantation sites could be harvested for histological studies.
REFERENCES


APPENDICES
APPENDIX A

STATISTICAL ANALYSIS

A.1 Shapiro-Wilk test for normality

Sample SAS programs and results

A.1.1. Sample 1

data svtrt;
input count @@;
output;
cards;
260 215 240 220 250 195;
ods listing close;
ods rtf file='odssvnormal_output.rtf';
proc univariate data=svtrt normal;
  var count;
run;
ods rtf close;
ods listing;

The SAS System
The UNIVARIATE Procedure
Variable: count

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<td>9.91631652</td>
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<tr>
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<td>Mode</td>
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<td>50% Median</td>
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<tr>
<td>25% Q1</td>
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<tr>
<td>10%</td>
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<tr>
<td>5%</td>
</tr>
<tr>
<td>1%</td>
</tr>
<tr>
<td>0% Min</td>
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</table>
A.1.2. Sample 2

data drugloaded;
input count @@;
output;
cards;
90 115 100 75 60 125
;
ods listing close;
ods rtf file='odsdrgnornal_output.rtf';
proc univariate data=drugloaded normal;
var count;
run;
ods rtf close;
ods listing;

The SAS System
The UNIVARIATE Procedure
Variable: count

<table>
<thead>
<tr>
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<td>75% Q3</td>
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</table>
A.2 Drug Comparison SMC proliferation study

A.2.1. Day 4

data;
input trt $ @;
do trial = 1 to 6;
input count @@;
output;
end;
cards;
cntl 345 325 300 290 210 290
sv  260 215 240 220 250 195
sva 95 70 45 75 30 75
;
ods listing close;
ods rtf file='drugday4_output.rtf';
proc print;
proc glm;
class trt;
model count = trt;
lsmeans trt /pdiff=all;
run;
ods rtf close;
ods listing;
### The SAS System

#### The GLM Procedure

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<td>sv</td>
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**Number of Observations Read** 18

**Number of Observations Used** 18
The SAS System
The GLM Procedure

Dependent Variable: count

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<th>Mean Square</th>
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<table>
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<tr>
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<th>Coeff Var</th>
<th>Root MSE</th>
<th>count Mean</th>
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### The SAS System
### The GLM Procedure
### Least Squares Means
### Adjustment for Multiple Comparisons: Tukey

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<tr>
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<th>LSMEAN Number</th>
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<tr>
<td>sva</td>
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</table>

### Least Squares Means for effect trt
**Pr > |t| for H0: LSMean(i)=LSMean(j)**

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<th>3</th>
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<tbody>
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<tr>
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<td>0.0122</td>
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<td>&lt;.0001</td>
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<tr>
<td>3</td>
<td>&lt;.0001</td>
<td></td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
A.2.2. Day 7

data;
input trt $ @;
do trial = 1 to 6;
in put count @@;
output;
end;
cards;
cntl  1225  1910  1660  1825  1680  1630
sv   1190  1030  1055  1210  1335  1290
sva  380   505  400  330  425  580
;
ods listing close;
ods rtf file='drugday7_output.rtf';
proc print;
proc glm;
class trt;
model count = trt;
lsmeans trt /pdiff=all;
run;
ods rtf close;
ods listing;
### The SAS System

#### The GLM Procedure

<table>
<thead>
<tr>
<th>Obs</th>
<th>trt</th>
<th>trial</th>
<th>Count</th>
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</thead>
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| Number of Observations Read | 18 |
| Number of Observations Used | 18 |
**The SAS System**

**The GLM Procedure**

*Dependent Variable: count*

<table>
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<tr>
<th>Source</th>
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<tbody>
<tr>
<td>Model</td>
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<tr>
<td>Corrected Total</td>
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<td>4926961.111</td>
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</tbody>
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<table>
<thead>
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<tr>
<td>0.919528</td>
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<td>4530477.778</td>
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**The SAS System**

**The GLM Procedure**

*Least Squares Means*

*Adjustment for Multiple Comparisons: Tukey*

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<th>LSMEAN Number</th>
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<tr>
<td>sva</td>
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</table>
A.3 Device Efficacy SMC proliferation study

A.3.1 Day 4

data;
input trt $ @;
do trial = 1 to 6;
input count @@;
output;
end;
cards;
cntl   260 260 320 395 265 335
sva    20 15 55 25 50 15
Dring  90 115 100 75 60 125
Bring  180 240 220 190 165 175;
ods listing close;
ods rtf file='deviceday4_output.rtf';
proc print;
proc glm;
class trt;
model count = trt;
lsmeans trt /pdiff=all;
run;
ods rtf close;
ods listing;
### The SAS System

<table>
<thead>
<tr>
<th>Obs</th>
<th>trt</th>
<th>trial</th>
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The GLM Procedure

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The SAS System
The GLM Procedure

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The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

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Pr > |t| for H0: LSMean(i)=LSMean(j)

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A.3.2. Day 7

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do trial = 1 to 6;
input count @@;
output;
end;
cards;
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sva 75 220 120 330 145 195
Dring 650 685 630 715 390 495
Bring 1105 1325 1000 1150 1215 1085;
ods listing close;
ods rtf file='deviceday7_output.rtf';
proc print;
proc glm;
class trt;
model count = trt;
lsmeans trt /pdiff=all;
run;
ods rtf close;
ods listing;
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#### The SAS System

#### The GLM Procedure

**Dependent Variable: count**

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**The GLM Procedure**  
**Least Squares Means**  
Adjustment for Multiple Comparisons: Tukey

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Pr > |t| for H0: LSMean(i)=LSMean(j)  
Dependent Variable: count

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APPENDIX B

PARTICLE SIZE ANALYSIS

B.1 Blank PLGA microspheres (o/w emulsion technique)

File name: AKSVB.sav
Sample ID: AKSVB
Operator: TR
Comments: in diluent; SV BLANK
Optical model: Fraunhofer
LS 100Q: Small Volume Module

Differential Volume (Average)

Volume Statistics (Arithmetic) aksvb.sav

Calculations from 0.375 \( \mu m \) to 648 \( \mu m \)

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<tr>
<td>Median</td>
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</tr>
<tr>
<td>D(3,2)</td>
<td>26.59 ( \mu m )</td>
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<tr>
<td>Mode</td>
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Volume Statistics (Arithmetic) Average of 3 files aksvb.sav

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APPENDIX C

CELL CULTURE PROTOCOLS

Thawing of Cells / Initiation of Culture
Process:
1. The recommended seeding density for AvsNC, BSMC, CASMC, PASMC, PrsMC, UASMC, BsdNC and UtsNC is 3,500 cells/cm².
2. To set up cultures calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used. Do not seed cells into a well plate directly out of cryopreservation. Add the appropriate amount of medium to the vessels (1 ml/6 cm²) and allow the vessels to equilibrate in a 37°C, 5% CO₂ humidified incubator for at least 30 minutes.
3. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, then rehydrate Quickly thaw the cryovial in a 37°C water bath being careful not to submerge the entire vial. Thawing the cells for longer than 2 minutes results in less than optimal results.
4. Resuspend the cells in the cryovial and using a micropipette, dispense cells into the culture vessels set up earlier. Gently rock the culture vessels to evenly distribute the cells and return to the incubator.
5. Centrifugation should not be performed to remove cells from cryoprotectant cocktail. This action is more damaging than the effects of DMPS residue in the culture.

Subculturing
The following instructions are for a 25 cm² flask. Adjust all volumes accordingly for other size flasks.

Preparation for subculturing the first flask:
1. Subculture the cells when they are 70 to 80% confluent and contain many mitotic figures throughout the flask.
2. For each 25 cm² of cells to be subcultured:
   a. Thaw 2 ml of Trypsin/EDTA and allow to come to room temperature.
   b. Allow 7-10 ml of HEPES Buffered Saline Solution (HEPES-BSS) to come to room temperature.
   c. Allow 4 ml of Trypsin Neutralizing Solution (TNS) to come to room temperature.
   d. Remove growth medium from 4°C storage and allow to start warming to room temperature.
   e. Prepare new culture vessels.
3. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol based on calculated cell count, cell viability, and seeding density.

In a sterile field:
1. Aspirate the medium from one culture vessel.
2. Rinse the cells with 5 ml of room temperature HEPES-BSS. DO NOT forget this step. The medium contains complex proteins and calcium that neutralize the trypsin.
3. Aspirate the HEPES-BSS from the flask.
4. Cover the cells with 2 ml of Trypsin/EDTA solution.
5. Examine the cell layer microscopically.
6. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. This entire process takes about 2 to 6 minutes, depending on cell type.
7. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, you may not have let them trypsinize long enough. Wait 30 seconds and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.
8. After cells are released, neutralize the trypsin in the flask with 4 ml of room temperature Trypsin Neutralizing Solution. If the majority of cells do not detach within seven minutes, the trypsin is either not warm enough or not active enough to release the cells. Harvest the culture vessels as described above, and either re-trypsinize with fresh, warm Trypsin/EDTA solution or rinse with Trypsin Neutralizing Solution and then add fresh, warm medium to the culture vessel and return to an incubator until fresh trypsinization reagents are available.
9. Quickly transfer the detached cells to a sterile 15 ml centrifuge tube.
10. Rinse the flask with a final 2 ml of HEPES-BSS to collect residual cells, and add this rinse to the centrifuge tube.
11. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%.
12. Centrifuge the harvested cells at 220 x g for 5 minutes to pellet the cells.
   a. Aspirate most of the supernatant, except for 100 to 200 µl.
   b. Flick the cryovial with your finger to loosen the pellet.
13. Dilute the cells in 2-3 ml of growth medium and note the total volume of the diluted cell suspension.

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14. Determine cell count and viability using a hemacytometer and Trypan Blue. Make a note of your cell yield for later use.

15. If necessary, dilute the suspension with the HEPES Buffered Saline Solution (HEPES-BSS) to achieve the desired "cells/ml" and re-count the cells.

16. Use the following equation to determine the total number of viable cells.

\[
\text{Total \# of Viable Cells} = \frac{\text{Total cell count \times percent viability}}{100}
\]

17. Determine the total number of flasks to inoculate by using the following equation. The number of flasks needed depends upon cell yield and seeding density. If seeding into well plates at this time, the recommended density is 10,000 cells/cm².

\[
\text{Total \# of Flasks to inoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area \times Rec. Seeding Density}}
\]

18. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

\[
\text{Seeding Volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of flasks as determined in step 18}}
\]

19. Prepare flasks by labeling each flask with the passage number, strain number, cell type and date.

20. Carefully transfer growth medium to new culture vessels by adding 1 ml growth medium for every 5 cm² surface area of the flask (1 ml/5 cm²).

21. After mixing the diluted cells with a 5 ml pipet to ensure a uniform suspension, dispense the calculated volume into the prepared subculture flasks.

22. If not using vented caps, loosen caps of flasks. Place the new culture vessels into a 37°C humidified incubator with 5% CO₂.

**Maintenance**

1. Change the growth medium the day after seeding and every other day thereafter. As the cells become more confluent, increase the volume of media as follows: under 25% confluence then feed cells 1 ml per 5 cm², 25-45% confluence then feed cells 1.5 ml per cm², over 45% confluence then feed cells 2 ml per 5 cm².

2. Warm an appropriate amount of medium to 37°C in a sterile container. Remove the medium and replace it with warmed, fresh medium and return the flask to the incubator.

3. Avoid repeated warming and cooling of the medium. If the entire contents are not needed for a single procedure, transfer only the required volume to a sterile secondary container.